UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/836,750	04/17/2001	James P. Elia	1000-10-C01	7239

7590 01/24/2008

Gerald K. White
GERALD K. WHITE & ASSOCIATES, P.C.
205 W. Randolph Street, Suite 835
Chicago, IL 60606

EXAMINER	
KEMMERER, ELIZABETH	
ART UNIT	PAPER NUMBER
1646	

MAIL DATE	DELIVERY MODE
01/24/2008	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

UNITED STATES PATENT AND TRADEMARK OFFICE

Commissioner for Patents
United States Patent and Trademark Office
P.O. Box 1450
Alexandria, VA 22313-1450
www.uspto.gov

BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

Application Number: 09/836,750
Filing Date: April 17, 2001
Appellant(s): ELIA, JAMES P.

Gerald K. White and Charles N. Lovell
For Appellant

EXAMINER'S ANSWER

This is in response to the appeal brief filed 23 February 2007, 08 June 2007, and 24 October 2007 appealing from the Office action mailed 22 September 2006.

(1) Real Party in Interest

A statement identifying by name the real party in interest is contained in the brief of 24 October 2007.

(2) Related Appeals and Interferences

The following are the related appeals, interferences, and judicial proceedings known to the examiner which may be related to, directly affect or be directly affected by or have a bearing on the Board's decision in the pending appeal:

While Appellant has referred to the Appeal Brief filed in 09/794,456 (in the brief of 24 October 2007), the examiner is aware of a number of applications claiming similar subject matter. They are as follows:

1) 09/064,000, "METHOD AND APPARATUS FOR INSTALLATION OF DENTAL IMPLANT' Under non-final rejection. Claims are **not directed to dental methods**, but rather are generally directed to methods of growing and integrating a desired soft tissue at a selected site in a body of a human patient wherein said desired soft tissue comprises a desired artery, comprising locally injecting stem cells into said body at said selected site, forming a bud at said selected site, and growing a new artery.

2) 10/179,589, "METHOD FOR GROWING HUMAN ORGANS AND SUBORGANS" Under non-final rejection. Claims generally directed to methods of growing an artery at a selected site in a body of a human patient comprising placing a stem cell in said body and growing a new artery.

3) 10/791,648, "TREATMENT FOR ARTHRITIS" Under final rejection. Claims generally directed to methods for treating arthritis comprising administering a stem cell at a desired location in a human patient to reduce inflammation and grow a blood vessel.

4) 11/605,152 "METHOD AND APPARATUS FOR INSTALLATION OF A DENTAL IMPLANT" Not yet examined. Claims are **not directed to dental methods**, but rather are generally directed to methods of repairing a dead portion of a pre-existing heart comprising administering stem cells and forming a new artery, which greatly overlap the subject matter of the instant claims.

5) 11/605,153 "METHOD AND APPARATUS FOR INSTALLATION OF A DENTAL IMPLANT" Not yet examined. Claims are **not directed to dental methods**, but rather are generally directed to methods of repairing a dead portion of a pre-existing

heart comprising administering stem cells and forming new cardiac muscle and a new artery.

6) 11/810,798 "METHODS FOR TREATING DISEASES AND INCREASING LONGEVITY" Not yet examined. Claims generally directed to methods of treating a brain in a patient having a damaged/dead portion of a brain caused by stroke, comprising administering stem cells to the brain and thus forming an artery, so that the brain portion is repaired.

7) 11/811,389 "METHOD AND APPARATUS FOR INSTALLATION OF A DENTAL IMPLANT" Not yet examined. Claims are **not directed to dental methods**, but rather are generally directed to methods for treating/rejuvenating/restoring function to a pancreas comprising administering stem cells to said pancreas and growing Islets of Langerhans and a new artery in said pancreas.

8) 11/891,456 "METHOD AND APPARATUS FOR INSTALLATION OF A DENTAL IMPLANT" Not yet examined. Claims are **not directed to dental methods**, but rather are generally directed to methods of producing and integrating soft tissue at a selected site of a human patient comprising placing stem cells in the body, forming a bud, and growing said soft tissue, preferably an artery.

(3) Status of Claims

The statement of the status of claims contained in the brief (of 24 October 2007) is correct.

(4) Status of Amendments After Final

The appellant's statement of the status of amendments after final rejection contained in the brief (of 24 October 2007) is correct. The after final amendment received 15 October 2007 is hereby entered by the examiner.

(5) Summary of Claimed Subject Matter

The summary of claimed subject matter contained in the brief (of 24 October 2007) is correct. Of course, the examiner disagrees with the assertions of patentability present in the summary, for reasons of record and as set forth below.

(6) Grounds of Rejection to be Reviewed on Appeal

The appellant's statement of the grounds of rejection to be reviewed on appeal is correct (in the Brief received 24 October 2007).

(7) Claims Appendix

The copy of the appealed claims contained in the Appendix to the brief (of 24 October 2007) is correct.

(8) Evidence Relied Upon

Strauer, B.E. et al. "Repair of Infarcted Myocardium by Autologous Intracoronary Mononuclear Bone Marrow Cell Transplantation in Humans" Clinical Investigations and Reports, vol106 (2002), pp. 1913-1918.

Murry, C.E. et al. "Skeletal Myoblast Transplantation for Repair of Myocardial Necrosis" Journal of Clinical Investigation, vol98, no. 11 (Dec 1996), pp. 2512-2523.

Klug, M.G. et al. "Genetically Selected Cardiomyocytes from Differentiating Embryonic Stem Cells Form Stable Intracardiac Grafts" Journal of Clinical Investigation, vol98, no. 1 (Jul 1996), pp. 216-224.

Oakley, R.M.E. et al. "Myocyte Transplantation for Myocardial Repair: A Few Good Cells Can Mend a Broken Heart" Annals of Thoracic Surgery, vol71 (2001), pp. 1724-1733.

Chiu, R.C.J. et al. "Cellular Cardiomyoplasty: Myocardial Regeneration With Satellite Cell Implantation" Annals of Thoracic Surgery, vol60 (1995), pp. 12-18.

Yoon, P.D. et al. "Myocardial Regeneration" Texas Heart Institute Journal, vol22 (1995), pp. 119-125.

Koh, G.Y. et al. "Differentiation and Long-Term Survival of C2C12 Myoblast Grafts in Heart" Journal of Clinical Investigation, vol92, (Sept 1993), pp. 1548-1554.

Van Meter, C. H. et al. "Myoblast Transplantation in the Porcine Model: A Potential Technique for Myocardial Repair" Journal of Thoracic and Cardiovascular Surgery, vol110, (1995), pp. 1442-1448.

Koh, G.Y. et al. "Targeted Expression of Transforming Growth Factor-β1 in Intracardiac Grafts Promotes Vascular Endothelial Cell DNA Synthesis" Journal of Clinical Investigation, vol95, (Jan 1995), pp. 114-121.

Wollert, K.C. et al. "Intracoronary autologous bone-marrow cell transfer after myocardial infarction: the BOOST randomised controlled clinical trial" Lancet, vol364, (2004), pp. 141-148.

Strauer, B.E. et al. "Stem Cell Therapy in Perspective" Circulation, vol107, (2003), pp. 929-934.

Deb, A. et al. "Bone Marrow-Derived Cardiomyocytes Are Present in Adult Human Heart : A Study of Gender-Mismatched Bone Marrow Transplantation Patients" Circulation, vol107, (2003), pp. 1247-1249.

5,328,470 NABEL et al. 7-1994

(9) Grounds of Rejection

The following ground(s) of rejection are applicable to the appealed claims:

35 U.S.C. § 112, First Paragraph, Enablement

The following is a quotation of the first paragraph of 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Claims 236, 238, 239, 243, 244, 247, 250, 251, 253, 257-263, 268-271, and 280-285 are rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the enablement requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention. The basis of this rejection is of record, but is re-printed here as per Appellant's request.

The claims require formation of a "new" artery. Appellant has defined a new artery as an organ comprising two or more kinds of tissues joined into one structure that has a certain task in the circulatory system. In Appellant's remarks section of the amendment received 17 February 2004, Appellant implies that the "new artery" recited in the claims must be formed *de novo*, and not merely repair, growth or re-direction of an existing artery. See the discussion regarding fusion versus formation of new cells.

The courts have determined several factors to be considered in making a determination of whether or not undue experimentation would have been required of the skilled artisan to make and use the claimed invention (*In re Wands*, 858 F.2d 731, 8 USPQ2d 1400 (Fed. Cir. 1988)). These are:

1) quantity of experimentation required,

2) amount of direction/guidance presented in the specification,

3) presence or absence of working examples,

4) nature of the invention,

5) state of the prior art,

6) level of skill of those in the art,

7) predictability, and

8) breadth of the claims.

1) In the instant case, the quantity of experimentation required would be very large. Appellant's attention is directed to pp. 1916 to 1918 of Strauer (of record, 2002, Circulation 106:1913-1918), who review the critical questions that had to be addressed while designing and realizing their trial of administering stem cells to human patients to repair damaged heart tissue. These included decisions regarding what cell population to use, what delivery method to use, and when cells should be transplanted. As can be seen from pp. 1916-1918, these were not simple or routine matters and involved great quantities of experimentation. In fact, one can see that the determinations of these details involved the act of invention.

2) The specification provides no guidance along the lines of the details worked out by Strauer. The specification broadly asserts that the administration of cells can achieve diverse effects, including growth of any "hard" tissue or "soft" tissue (p. 20), formation of entire new organs (p. 32) or portions of organs (p. 46), restoration of function in any organ (p. 47), formation of auxiliary organs (p. 49), correction of necrosis (p. 49), replacement of missing limbs or body parts (p. 50), treatment of inflammation (p. 50), correction of musculoskeletal injuries or deficiencies (p. 50), formation of hybrid organs (p. 50), etc. No guidance or details are provided as to *how* to achieve these

remarkable effects, most of which have never been achieved in this art to this day. The courts have stated that "[p]atent protection is granted in return for an enabling disclosure of an invention, not for vague intimations of general ideas that may or may not be workable". Genentech Inc. v. Novo Nordisk A/S (CAFC) 42 USPQ2d 1001 (1997). The courts have also stated that "[t]ossing out the mere germ of an idea does not constitute an enabling disclosure... [R]easonable detail must be provided in order to enable members of the public to understand and carry out the invention" (Genentech Inc. v. Novo Nordisk A/S, *supra*).

3) The specification contains only prophetic examples. In fact, none of the prophetic examples are directed to administration of cells to grow a new artery, thus repairing a dead or damaged portion of a heart. Therefore, there are no examples, working or prophetic, directed to the elected invention.

4) The nature of the invention is highly complex, as evidenced by all of the publications of record, including Strauer et al. 2002. All inventions involving administration of active agents of any kind to a patient to achieve a physiological reaction are complex. See also Murry et al. (of record, 1996, J. Clin. Invest. 98:2512-2523), who state that "the goal of limiting myocardial injury has been difficult to achieve clinically, because ischemic myocardium dies quite rapidly and most patients wait more than 3 h after coronary occlusion before seeking medical attention" (p. 2512, Introduction).

5) The state of the art does not support the specification's (and claims') assertion that a **new** artery can be grown. None of the numerous post-filing date publications put

on the record by Appellant to support enablement of the claimed invention report the *de novo* growth of an artery as defined by Appellant, including Strauer et al. 2002. Furthermore, whereas none of the claims except for 270 and 271 require localized administration at the site of dead or damaged myocardium, the state of the prior art indicates that only localized injection of cells could successfully treat damaged myocardium for *any* positive effect. See Murry et al. (*supra*), Klug et al. (1996, J. Clin. Invest. 98:216-224), Oakley et al. (2001, Ann. Thorac. Surg. 71:1724-1733), Chiu et al. (1995, Ann. Thorac. Surg. 60:12-8), Yoon et al. (1995, Tex. Heart Inst. J. 22:119-125), Koh et al. (1993, J. Clin. Invest. 92:1548-1554), Van Meter et al. (1995, J. Thorac. Cardiovasc. Surg. 110:1442-1448), and Koh et al. (1995, J. Clin. Invest. 95:114-121). All used intramuscular injection of cells directly into the myocardium. However, none of these references show evidence of growth of a new artery as required by the claims.

6) The level of skill in the art is admittedly high.

7) The invention is unpredictable, as it involves administering active agents to a living patient to achieve a physiological response. As was found in Ex parte Hitzeman, 9 USPQ2d 1821 (BPAI 1987), most chemical reactions and physiological activity involve unpredictable factors. See also In re Fisher, 427 F.2d 833, 839, 166 USPQ 18, 24 (CCPA 1970); Amgen Inc. v. Chugai Pharmaceutical Co. Ltd., 927 F.2d 1200, 1212, 18 USPQ2d 1016, 1026 (Fed. Cir.), cert. denied, 502 U.S. 856 (1991).

8) The breadth of the claims is quite large. The elected invention is directed to a method of administering any type of cell to an undefined area of a human body (not limited to the dead/damaged heart area except for claims 270 and 271) to grow new

cardiac muscle and a new artery (of any type or location) to achieve growth of a new portion of a pre-existing heart.

Due to the large quantity of experimentation necessary to determine how to effectively administer cells to achieve *de novo* formation of cardiac muscle and an artery and thereby grow a new portion of a pre-existing heart, the lack of direction/guidance presented in the specification regarding the same, the absence of working examples directed to the same, the complex nature of the invention, the contradictory state of the prior art, the unpredictability of the effects of an agent on a physiological response, and the breadth of the claims which fail to recite limitations regarding cell type or dosage or site of delivery, etc., undue experimentation would be required of the skilled artisan to make and/or use the claimed invention in its full scope.

(10) Response to Argument

At pp. 9-10 of the Appeal Brief of 24 October 2007, Appellant argues that consideration of three factors leads to the conclusion that the discourse is enabling. Appellant characterizes the first factor as the existence of a considerable body of disclosure relating to Appellant's generic invention of repairing organs in human patients, including heart, by forming new cardiac muscle and a new artery and of elected and non-elected, well-known growth factors suitable for achieving such repair. Appellant urges that the examiner's selective reading of the disclosure as it relates to the elected growth factor, cells, is erroneous. Appellant quotes from the 22 February 2006 Office Action in copending application 09/794,456 wherein the examiner stated that the generic concept of growth factor is not relevant. Appellant also refers to pp. 10

and 12 of the final rejection of 22 September 2006 in the instant application as evidence that the examiner erroneously ignored disclosure related to non-elected species. This has been fully considered but is not found to be persuasive. The 22 February 2006 quote used by Appellant was taken from a different rejection (new matter) of claims that Appellant subsequently canceled in a different application, and thus is taken completely out of context. Regarding the 22 September 2006 final rejection in the instant application, p. 10 specifically is directed to issues concerning the elected invention. Page 12 refers to issues concerning two words that no longer appear in any of the claims on appeal. Therefore, these sections of the office action are also not improper and irrelevant to the rejection being appealed. Furthermore, of the 26 claims Appellant has chosen to appeal, 11 specifically require administration of cells, and thus it was proper to consider enablement of administration of cells to achieve the results required by the claims. Finally, Appellant's characterization of the specification as constituting a "substantial body of disclosure" is unsupported in this section of the arguments, and is thus not found to be persuasive. Arguments of counsel alone cannot take the place of evidence in the record once an examiner has advanced a reasonable basis for questioning the disclosure. See *In re Budnick*, 537 F.2d at 538, 190 USPQ at 424; *In re Schulze*, 346 F.2d 600, 145 USPQ 716 (CCPA 1965); *In re Cole*, 326 F.2d 769, 140 USPQ 230 (CCPA 1964). None of the evidence on record demonstrates formation of a new artery, for example.

At p. 11 of the Appeal Brief of 24 October 2007, Appellant argues that the second factor to consider is that the examiner has not taken issue with the fact that the

administration techniques and materials were old and well known at the time of the filing date. Appellant characterizes the inventor's contribution to the medical arts as the combination of using the old materials and techniques to achieve a new result. This has been fully considered but is not found to be persuasive. The issue is that the instant specification does not teach the skilled artisan *how* to manipulate these allegedly old materials and methods to achieve remarkable effects. The instant specification does not exemplify nor provide detailed guidance as to *how* a single organ, part of an organ, tissue, artery, or even a bud can be formed by merely placing cells in a body. Appellant claims to have achieved something no one else had done simply by writing it down. To say that non-obvious and remarkable results can be achieved without doing a single experiment is incredible. It is a remarkable achievement to grow a new artery by implanting cells, but Appellant did not do it and Appellant's disclosure does not teach anyone how to do it.

At p. 11 of the Appeal Brief of 24 October 2007, Appellant characterizes the third factor as the high level of skill in the art. Appellant urges that many years of education, training, and experience are required in the medical field. The examiner takes no issue with this statement. Indeed, such a level of skill is required of anyone conducting the type of biological research and therapy that is the subject matter of the instant claims. However, the level of skill in the art is only one of the factors set forth by the court in *In re Wands*, 858 F.2d 731, 8 USPQ2d 1400 (Fed. Cir. 1988)). It is improper to conclude that a disclosure is not enabling (or, conversely, enabling) based on an analysis of only one of the factors while ignoring one or more of the others. The examiner's analysis

must consider all the evidence related to each of these factors, and any conclusion of nonenablement must be based on the evidence as a whole. 858 F.2d at 737, 740, 8 USPQ2d at 1404, 1407. In the instant application, the preponderance of all of the evidence concerning all eight factors (quantity of experimentation required, amount of direction/guidance presented in the specification, presence or absence of working examples, level of skill in the art, nature of the invention, state of the prior art, predictability, and breadth of the claims) led to the conclusion that the claimed methods are not enabled.

At pp. 11-12 of the Appeal Brief of 24 October 2007, Appellant refers to M.P.E.P. § 2164.01 as indicating that detailed procedures for making and using the invention may not be necessary if the description of the invention itself is sufficient to permit those skilled in the art to make and use the invention. Appellant again asserts that the materials and administration techniques are old, and that one skilled in the medical arts would be enabled to make and use the claimed invention. This has been fully considered but is not found to be persuasive. Again, the issue is that the instant specification does not teach the skilled artisan *how* to manipulate these allegedly old materials and methods to achieve the remarkable effects required by the claims (growth of new cardiac muscle, growth of a new artery, repair of dead/damaged heart tissue). The claims merely recite placing a growth factor such as a cell at an undefined selected area and waiting for new cardiac muscle and a new artery to grow and dead/damaged heart tissue to be repaired. The post-filing date art provides evidence of the further act of invention that was required to achieve any positive results (e.g.; Strauer et al. 2002,

pp. 1916-1918). It is also telling that none of the art of record, including Strauer et al. 2002, has achieved formation of a new artery.

At p. 13 of the Appeal Brief of 24 October 2007, Appellant argues that the examiner has failed to meet the burden of establishing a *prima facie* case of lack of enablement supported by convincing objective evidence. Appellant refers to the conclusory paragraph of the enablement rejection set forth in the final rejection of 22 September 2006, and argues that the conclusion is of an improper conditional nature regarding the *de novo* formation of cardiac muscle and arteries. Appellant urges that *de novo* formation of cardiac muscle and arteries is not required of the claims. This has been fully considered but is not found to be persuasive. Each independent claim recites "growing **new** cardiac muscle and growing a **new** artery" (emphasis added). The translation of "*de novo*" is "anew." Thus, the claims are precisely requiring *de novo* formation of cardiac muscle and arteries.

At pp. 13-14 of the Appeal Brief of 24 October 2007, Appellant argues that the examiner's construction of the claims is erroneous. Appellant takes issue with the examiner's interpretation of "forming a new artery" as being limited to *de novo* formation of an artery, and not merely repair, growth, or re-direction of an existing artery. Appellant refers to p. 44 of the specification as defining the term "organ" and pp. 45-50 as disclosing that growth factors may be used to grow and form new muscles and arteries. Appellant refers to pp. 54, 56, and 62 of the specification as clearly defining the term "new artery." At the paragraph bridging pp. 14-15 of the brief, Appellant urges that the examiner has improperly become the lexicographer. This has been fully considered

but is not found to be persuasive. For clarity, the relevant sections of pp. 54, 56, and 62 of the specification are reproduced here:

p. 54:

"After four weeks, another MRI is taken which shows the patient's leg artery. The MRI shows that (1) at the first site a new artery is growing adjacent the patient's original leg artery, and (2) at the second site a new section of artery is growing integral with the original artery, i.e., at the second site the new section of artery is lengthening the original artery. much like inserting a new section of hose in a garden hose concentric with the longitudinal axis of the garden hose lengthens the garden hose."

This section clearly supports the examiner's interpretation, since the specification distinguishes between growth of a new artery adjacent to an existing artery and growing a new *section* of an artery integral with the original artery. The instant claims recite "growing a new artery" and not "growing a new *section of* an artery."

p. 56:

"Anatomic evidence of collateral artery formation is observed by the 30th day following injection to the RAOTS construct. One end of the artery integrates itself in the heart wall to receive blood from the heart. The other end of the artery branches into increasing smaller blood vessels to distribute blood into the heart muscle. Once the growth of the new artery is completed, the new artery is left in place in the heart wall. Transplantation of the new artery is not required."

This section is also consistent with the examiner's interpretation. The "new artery" is not described in this section as being integral with an existing artery, but rather is described as integrating one end into the heart.

p. 62:

Similar results are obtained, i.e., a new section of artery grows integral with the original artery, and a new section of artery grows adjacent the original artery. The new section

of artery has integrated itself at either end with the original artery so that blood flows through the new section of artery.

Again, this is consistent with the examiner's interpretation. The specification at p. 62 refers to new *sections* of arteries, not "new artery" as recited in the claims. Finally, it is noted that the claims were amended to recite "growing a *new* artery" in the amendment of 17 February 2004 so as to further distinguish the claimed invention from the disclosure of Murry et al. to overcome a rejection under 35 U.S.C. § 103(a) over Murry et al. (see p. 39 of the remarks section). Since Murry et al. disclosed growth of new capillaries, presumably by extension from (and integral with) existing arteries, Appellant's amendment was taken as distinguishing the claims from blood vessel growth from existing arteries in an integral fashion. Considering all of the evidence, the interpretation of the claims is reasonable and supported by the evidence of record.

At pp. 15-16 of the Appeal Brief of 24 October 2007, Appellant reviews the legal requirements of objective enablement with reference to case law, with which the examiner takes no issue.

At p. 16 of the Appeal Brief of 24 October 2007, Appellant states that the examiner only addressed the specifically claimed subject matter in claims 248, 249, and 274-277, which Appellant has just canceled in the after final amendment of 15 October 2007. Appellant argues that the examiner's discussion of Strauer et al. 2002, Deb et al. and Murry et al. are not relevant to injection of stem cells and growing an artery as required by claim 268 and 269. This has been fully considered but is not found to be persuasive since it is factually incorrect. Consideration of all of the claims was provided

in the analysis on pp. 7-11 of the final rejection (of 22 September 2006) with a conclusion section at the second paragraph of p. 11. After that, further enablement issues pertinent to claims 248, 249, and 274-277 were addressed. Strauer et al. 2002 and Murry et al. are still relevant to the claimed invention in that successful treatment of damaged myocardium was achieved in those publications, and they speak to the type of experimentation that had to occur before such success, and serve as a contrast to the instant specification's dearth of detailed guidance. Deb et al. speaks to the insufficient numbers of cells that will migrate to the heart when administered to a site distant from the heart, and thus also speaks to the lack of enablement of any of the claims which do not require local administration. Also, none of Strauer et al. 2002, Deb et al., or Murry et al. achieved formation of a new artery despite administration of cells, further providing evidence of non-enablement.

At pp. 16-18 of the Appeal Brief of 24 October 2007, Appellant states that the examiner failed to consider the specification as a whole and the amount of disclosure in the prior art. Appellant urges that analysis of the Wands factors leads to a conclusion of enablement. Appellant argues that Strauer et al. 2002 does not disclose any experimental protocol required for practicing the invention. Appellant indicates that Strauer et al. 2002 used an off-the-shelf angioplasty balloon catheter such as that disclosed in US 5328470 (Nabel et al.). Appellant indicates that Nabel et al. also provide guidance regarding back flow prevention and contact time. Appellant concludes that Strauer et al. 2002 did not have to perform any experimentation. This has been fully considered but is not found to be persuasive. Strauer et al. 2002 administered 6 to

7 fractional high-pressure infusions of 2 to 3 mL cell suspension, each of which contained 1.5 to 4 X 10^6 mononuclear cells directly to the infarct site in order to achieve repair of damaged heart tissue. Strauer et al. 2002 specifically point out what obstacles had to be overcome at pp. 1916-1918 in great detail. Their success led to publication in a prominent, peer-reviewed journal. Numerous other post-filing date publications are cited by Strauer et al. 2002 at pp. 1916-1918. Nowhere do Strauer et al. refer to Nabel et al. Furthermore, nowhere do Nabel et al. report the type of experimental detail provided by Strauer et al. 2002 or growth of a new artery or repair of dead/damaged heart tissue, despite disclosing placement of cells at a selected area of a patient with ischemic heart, which inherently comprises a dead/damaged portion (see abstract and col. 2, li.40-50). Thus, Nabel et al. also constitutes evidence of non-enablement of the instant claims. Considering the differences between Nabel et al. and Strauer et al. 2002, it is clear that Strauer et al. 2002 provide the information as to *how* to achieve repair of heart tissue by administration of cells, whereas Nabel et al. (and the instant specification) do not. None of the art of record achieves formation of new cardiac muscle and a new artery, despite the fact that some of them report administration of cells to a patient with a damaged heart, thus providing further evidence of non-enablement. The courts have stated that "[p]atent protection is granted in return for an enabling disclosure of an invention, not for vague intimations of general ideas that may or may not be workable". Genentech Inc. v. Novo Nordisk A/S (CAFC) 42 USPQ2d 1001 (1997). The courts have also stated that "[t]ossing out the mere germ of an idea does not constitute an enabling disclosure... [R]easonable detail must be provided in

order to enable members of the public to understand and carry out the invention" (Genentech Inc. v. Novo Nordisk A/S, *supra*). The instant specification does not provide an enabling disclosure in accordance with the guidance set forth in these decisions.

At p. 18 of the Appeal Brief of 24 October 2007, Appellant argues that the specification provides direction and guidance for use of a containment system for controlling carry-away and prolonged contact of cells in Examples 18 and 19 by cell injection. Appellant stresses that claims 247, 250, 268, and 269 recite injection of cells. This has been fully considered but is not found to be persuasive. The only reference to a containment system are the two sentences in prophetic example 18 which states "A containment system is placed at the first site" and "0.25 milliliters of the genetic carrier solution is injected into the containment system at the first site." No details regarding the structure or even the precise function of the containment system is given. Prophetic example 19 does not mention a containment system at all. This is hardly direction and guidance for using a containment system. Also, the skilled artisan would not assume that any containment system for containing nucleic acids would be the same as one used for containing cells, proteins, extracellular matrices, sources of energy, or anything else that Appellant indicates is encompassed by the term "growth factor." Furthermore, both prophetic examples 18 and 19 state that injection must be performed "slowly" to avoid leakage to the interior of an existing artery or through an external covering of muscle cells. However, no details or guidance are given regarding how to achieve slow injection, or just how slow the injection must be in order to achieve the desired results. Basically, the skilled artisan is provided with a suggestion and then is invited to

experiment to achieve results that Appellant characterizes as "remarkable." Finally, both prophetic Examples 18 and 19 are directed to injection of nucleic acids, whereas none of claims 247, 250, 268, or 269 are limited to such. Some of the claims are limited to injection of cells (claims 243, 244, 253, 260-263, 268-271; *not* claims 247 and 250 as stated incorrectly by Appellant). Any example, real or prophetic, involving administration of nucleic acids provides no guidance regarding administration of cells. The scientific considerations of handling, dosage, carriers, etc. are completely different. The other claims under appeal are much more broadly drawn to injection of "growth factors" which Appellant appears to indicate in the specification as encompassing any substance or source of energy that promotes growth. Surely two prophetic examples of administration of nucleic acids are not commensurate in scope with such vast claims. In fact, the enormous breadth of these claims is a factor supporting the instant enablement rejection.

Also at p. 18 of the Appeal Brief of 24 October 2007, Appellant urges that the examiner has conceded that administration of cells is old in the art at p. 22 of the final rejection. This has been fully considered but is not found to be persuasive because, while administration of cells is old (for example, in blood transfusion methods), administration of cells to achieve new cardiac muscle growth and new artery growth in combination with repair of damaged/dead heart tissue as required by the claims has not been achieved to date.

Also at pp. 18-19 of the Appeal Brief of 24 October 2007, Appellant argues that Strauer et al. 2002 do not describe using any experimental protocol to determine

appropriate cell population. Appellant urges that there is no requirement for using specific bone marrow stem cell species. This has been fully considered but is not found to be persuasive. Strauer et al. 2002 state clearly and in detail that cell population is critical at pp. 1916-1917. In peer-reviewed journal articles, failed experiments are generally not reported, and thus when the successful regimen is disclosed, it cannot be concluded that no experimentation was done. Appellant has provided no evidence that other cells would achieve new cardiac muscle growth or new artery growth and/or repair of dead/damaged heart tissue. It must be emphasized that arguments of counsel alone cannot take the place of evidence in the record once an examiner has advanced a reasonable basis for questioning the disclosure. See *In re Budnick*, 537 F.2d at 538, 190 USPQ at 424; *In re Schulze*, 346 F.2d 600, 145 USPQ 716 (CCPA 1965); *In re Cole*, 326 F.2d 769, 140 USPQ 230 (CCPA 1964). For example, in a case where the record consisted substantially of arguments and opinions of Appellant's attorney, the court indicated that factual affidavits could have provided important evidence on the issue of enablement. See *In re Knowlton*, 500 F.2d at 572, 183 USPQ at 37; *In re Wiseman*, 596 F.2d 1019, 201 USPQ 658 (CCPA 1979). In the instant case, a reasonable basis for questioning the disclosure has been established of record, and thus the burden of evidence has shifted to Appellant to provide evidence.

Finally, at pp. 18-19 of the Appeal Brief of 24 October 2007, Appellant argues that Strauer et al. 2002 do not disclose that timing of treatment required experimentation. Appellant points to Strauer et al. 2005 as showing that patients having suffered MI can be treated as long as 27 months after MI. Appellant concludes that the

examiner's conclusion that great quantities of experimentation would be required is "fatally flawed." This has been fully considered but is not found to be persuasive. The conclusion that a great quantity of experimentation would have been required was not based on time of administration alone. See Office Action mailed 22 September 2006, p. 9, for example. The type of cell administered as well as administration methods were also deemed to be critical in the art to achieve repair of damaged heart tissue. None of the art has determined the conditions necessary to achieve formation of new cardiac muscle and a new artery, and thus more experimentation is required to overcome this failing. Consideration of all of the evidence pertinent to this factor along with the other factors reasonably leads to the conclusion of non-enablement.

At p. 19 of the Appeal Brief of 24 October 2007, Appellant argues that the examiner's contention that the specification fails to provide guidance along the lines of the details worked out by Strauer et al. 2002 is misplaced. Appellant urges that none of the appealed claims require an angioplasty balloon catheter, and that example 19 describes a detailed regimen for treating a patient with a damaged heart by injecting a growth factor for promoting artery growth. Appellant also points to pp. 40-42, 47, and 48 as providing guidance for use of autologous stem cells harvested from bone marrow and blood of the patient or from cell cultures to grow organs. This has been fully considered but is not found to be persuasive. The only evidence of record of the achievement of repair of damaged heart tissue is by angioplasty delivery of specific stem cells such as in Strauer et al.'s 2002 publication. No evidence has been submitted to show growth of new cardiac muscle and a new artery by any research group. It is

important to keep in mind that Appellant is attempting to rely upon the Strauer et al. publications as evidence supporting enablement of the instant claims. Strauer et al. 2002 and 2005 are not at all commensurate in scope with the instant claims which broadly read on "placement" of growth factors in an undefined place in a body of a patient, growing new cardiac muscle and a new artery, wherein the claims do not recite any procedure to achieve new cardiac muscle growth and new artery growth other than observation. Strauer et al. 2002 and 2005 make it clear that there are many experimental details that had to be worked out in order to achieve any positive effect on damaged heart tissue. Such details were not disclosed in the instant specification and involved the further act of invention. Furthermore, not even Strauer et al. 2002 achieved growth of new cardiac muscle and a new artery. Therefore, the preponderance of the totality of the evidence supports the instant enablement rejection. Regarding prophetic example 19, the disclosure is limited to details for injection of nucleic acid, which is not the specific subject matter of any of the claims under appeal. Furthermore, the prophetic example is greatly lacking in detail. Regarding pp. 40-42, 47, and 48, prophetic example 11 mentions states that living stem cells are harvested from bone marrow, blood, or cell culture, but does not state how or what type of stem cells are isolated, or to what purpose. Prophetic example 14 mentions stem cells, indicates that they are "starved," although how or why this is done is not explained, and further states that an electric spark is applied to the culture medium, again without an explanation of how or why. Prophetic example 15 states that stem cells are harvested from bone marrow, blood or cell culture, apparently mixed with WT-1 and PAX genes,

BMP-7 and other kidney BMPs in order to produce a primitive kidney germ. Again, this prophetic example is devoid of even basic details such as which BMPs are used or how the conditions are manipulated to produce a "kidney germ." Prophetic example 16 is much like prophetic example 15 except that Aniridia transcription factor" and growth factors and the Aniridia gene are added to the culture medium to produce an "eye germ." Again, this prophetic example is devoid of even basic details such as which growth factors are used or how the conditions are manipulated to produce a "eye germ." Pages 47-48 state that a skin cell from the lining of a cheek can be isolated, screened for DNA damage, repaired if DNA damage is detected, dedifferentiated in culture and then redifferentiated to form an organ. Such would truly be a remarkable achievement. However, the specification states that it *can* be done, but does not provide even basic guidance as to *how* it can be done. No culture conditions are described, for example, to achieve dedifferentiation and redifferentiation.

From pp. 19 to 20 of the Appeal Brief of 24 October 2007, Appellant argues that working examples are not required for enablement. Appellant refers to M.P.E.P. § 2164.02 as providing for prophetic examples. Appellant further argues that the examiner's position that none of the prophetic examples are directed to administering cells to grow a new artery is incorrect. Appellant refers to pp. 46-48 and 33 to support this last argument. This has been fully considered but is not found to be persuasive. M.P.E.P. § 2164.02 states that "the specification need not contain an example if the invention is otherwise disclosed in such a manner that one skilled in the art will be able to practice it without an undue amount of experimentation. *In re Borkowski*, 422 F.2d

904, 908, 164 USPQ 642, 645 (CCPA 1970). **Lack of a working example, however, is a factor to be considered, especially in a case involving an unpredictable and undeveloped art.**" (emphases added) In the instant case, the claimed invention is directed to an unpredictable and undeveloped art. As was found in Ex parte Hitzeman, 9 USPQ2d 1821 (BPAI 1987), a single embodiment may provide broad enablement in cases involving predictable factors such as mechanical or electrical elements, but **more will be required** in cases that involve **unpredictable factors such as most chemical reactions and physiological activity**. See also In re Fisher, 427 F.2d 833, 839, 166 USPQ 18, 24 (CCPA 1970); Amgen Inc. v. Chugai Pharmaceutical Co. Ltd., 927 F.2d 1200, 1212, 18 USPQ2d 1016, 1026 (Fed. Cir.), cert. denied, 502 U.S. 856 (1991). Furthermore, in the instant application, there is not a single example, working or prophetic that is directed to administration of a cell to result in growth of new cardiac muscle and a new artery in a pre-existing heart. Pages 46-48 and 33 do not describe this method. Page 46 states that cells may be use to grow new muscle in a heart, but does not explain how. Furthermore, page 46 states that genes would be required to grow new arteries (p. 46, li. 4-6). Pages 47-48 state that a skin cell from the lining of a cheek can be isolated, screened for DNA damage, repaired if DNA damage is detected, dedifferentiated in culture and then redifferentiated to form an organ, such as a new artery. Again, while such would truly be a remarkable achievement, the specification states that it *can* be done, but does not provide even basic guidance as to *how* it can be done. No culture conditions are described, for example, to achieve dedifferentiation and redifferentiation. Furthermore, this section of the specification does not link the growth

of new cardiac muscle or a new artery to repair of dead/damaged heart tissue. Page 33 does not appear to mention cells, arteries or heart tissue.

At p. 20 of the Appeal Brief of 24 October 2007, Appellant agrees with the examiner that the medical arts are complex. Appellant urges that the practice of the claimed invention is straightforward since it involves stem cells, methods of administering, and apparatus that are old and well-known in the medical arts. This has been fully considered but is not found to be persuasive. Appellant appears to take the position that the inventor has combined old products and methods to achieve remarkable results merely by writing it down in a new way. To achieve remarkable results in the "medical arts" or any field of biological research without the performance of any experimentation strains credulity. Consideration of the totality of the evidence pertaining to all eight of the Wands factors clearly leads to a conclusion of non-enablement.

At pp. 20-21 of the Appeal Brief of 24 October 2007, Appellant quotes from the final rejection and takes issue with the examiner's statement that Appellant has relied upon post-filing date art to support enablement of the claimed invention. Appellant states that the instant specification is relied upon for enablement. Appellant also argues that the examiner misunderstands the meaning of "state of the art" and characterizes it as being limited to whether materials and methods were known in the art. This has been fully considered but is not found to be persuasive. If Appellant is not relying on Strauer et al. 2002 as supporting enablement, then the examiner is at a loss as to why Appellant continues to refer to it. Appellant put this art on the record; not the examiner.

At pp. 8-9 of the remarks submitted with the amendment of 17 June 2003 in related application 09/794,456, Appellant stated, "Regarding evidence supporting the operability of claim 7, i.e., the repair of a dead portion of a heart, Appellant cited the Strauer publication in the concurrently filed Fourth Supplemental Information Disclosure Statement ("IDS"). Such publication discloses the use of a growth factor to repair a damaged, including dead, portion of a heart and thus provides strong evidence that that [sic] the methods of claims 7 and 21 are operable." Since this was submitted in response to the rejection of claim 7 under 35 U.S.C. § 101 for lack of utility and 35 U.S.C. § 112, first paragraph, for the accompanying rejection for lack of enablement, it is submitted that the record fairly indicates that Appellant has relied upon Strauer et al. 2002 as evidence in support of the enablement, at least in part. Regarding the meaning of "state of the art," M.P.E.P. § 2164.05 states, "The state of the prior art is what one skilled in the art would have known, at the time the application was filed, about the subject matter to which the claimed invention pertains." The M.P.E.P. does not indicate that such consideration is to be limited to materials and methods, but to the claimed invention as a whole. M.P.E.P. § 2164.05 further states, "The state of the art provides evidence for the degree of predictability in the art and is related to the amount of direction or guidance needed in the specification as filed to meet the enablement requirement. The state of the art is also related to the need for working examples in the specification. The state of the art is not static in time...the state of the art must be evaluated for each application based on its filing date." Again, this direction is not limited to consideration of materials and methods. It is noted that the instant application

claims benefit under 35 U.S.C. § 120 to 27 April 1993, arguably when stem cell therapy for purposes other than hematopoiesis was still in its infancy.

At p. 21 of the Appeal Brief of 24 October 2007, Appellant indicates that the examiner and Appellant are in agreement that the level of skill in the art was high at the time the instant application was filed. The examiner takes no issue with this statement.

At p. 21 of the Appeal Brief of 24 October 2007, Appellant argues that the examiner fails to provide any succinct reasoning as to why one skilled in the art would doubt that the asserted scope of objective enablement in the specification is not commensurate with the scope of the claims. Appellant asserts that the specification provides multiple embodiments using multiple well-known administration modes carried out with multiple well-known apparatus. This has been fully considered but is not found to be persuasive. The record has been fully developed concerning the evidence of record pertinent to the Wands factors. The Office Actions have painstakingly addressed every piece of relevant evidence. The preponderance of the totality of the evidence supports the enablement rejection. Without repeating all of the Office Actions in the instant examiner's answer, the examiner can only point to the record in response to Appellant's assertion that no reasoning has been provided. If the reasoning can be summed up briefly, it would be to state that while the specification states that old materials and old methods *can* be used to achieve "remarkable" results, it does not provide guidance as to *how* said remarkable results can be achieved.

At pp. 21-22 of the Appeal Brief of 24 October 2007, Appellant takes issue with the examiner's assessment of the breadth of the claims. Appellant argues that the

claims in issue require cells, stem cells, and bone marrow stem cells. Appellant urges that one of ordinary skill in the art appraised of the specification disclosure would readily comprehend the type of cell, "i.e., stem cell," required for promoting morphogenesis. This has been fully considered but is not found to be persuasive. Appellant now appears to assert that stem cells are all that is required. Previously in the arguments, Appellant urged that the examiner improperly dismissed those portions of the specification that provided guidance regarding other types of growth factors. Appellant appears to want it both ways. In the end, the broadest claims merely recite "growth factor" which is broadly defined in the specification as any factor that promotes growth. Cells, proteins, genes, extracellular matrices, sources of energy, living organisms, etc. are all encompassed by the term, according to the specification. See pp. 20-21, for example. However, even the narrowest claims merely recite a stem cell harvested from bone marrow. This is still broader than the type of cells the post-filing date art used to achieve repair of damaged heart tissue. For example, Strauer et al. 2002 used autologous mononuclear bone marrow cells.

At p. 22 of the Appeal Brief of 24 October 2007, Appellant argues that the claims require growing new cardiac muscle and a new artery which limits the selected placement area to those that result in cardiac muscle growth and artery growth in the patient's heart. Appellant indicates that the examiner's evaluation of the specification was "limited" and erroneously did not consider enablement provided by the specification as a whole. This has been fully considered but is not found to be persuasive. The specification, and Appellant's previous arguments of record, appear to indicate that

placement of any type of growth factor at any location would result in new cardiac growth and new artery growth in a heart. For example, Appellant has relied on Deb et al. 2003 as establishing that intravenous administration of cells results in migration of the cells to the heart to achieve the desired results. See pp. 32-33 of the remarks section of the amendment of 17 February 2004, for example. The broadest claims merely recite placement of a growth factor "in a body of a human patient." The claims are not limited to a site at or near the heart. No erroneous evaluation of the specification has been conducted. Furthermore, the specification provides no guidance regarding what site would result in formation of new cardiac muscle and a new artery, as this has not been achieved to date.

Also at p. 22 of the Appeal Brief of 24 October 2007, Appellant argues that the examiner failed to specifically address the inventions of claims 270 and 271 requiring placement of cells adjacent dead or damaged heart portions, and the inventions of claims 268 and 269 requiring injecting cells directly into the patient. This has been fully considered but is not found to be persuasive. It is noted that "adjacent" is a relative term. Lungs can be considered to be located adjacent to a heart. By "adjacent," are the claims limited to the healthy heart muscle next to the necrotic area, or a coronary blood vessel, or the pericardium, or the chest cavity as a whole? Similarly, injection "into" the patient can mean injection into any part of the patient. Thus, it is still submitted that these claims are rather broad in scope regarding site of placement. Also, Strauer et al. 2002 is not commensurate in scope with these claims if Strauer et al. 2002 is to be

relied upon as supporting enablement. Strauer et al. 2002 used high pressure infusion of cells into the infarct-related artery.

From pp. 22-23 of the Appeal Brief of 24 October 2007, Appellant again argues that the claims do not require *de novo* formation of a new artery, and thus the examiner's characterization of such as the basis of the rejection must fail. This has been fully considered but is not found to be persuasive. As discussed above, p. 54 of the specification reads:

"After four weeks, another MRI is taken which shows the patient's leg artery. The MRI shows that (1) at the first site a new artery is growing adjacent the patient's original leg artery, and (2) at the second site a new section of artery is growing integral with the original artery, i.e., at the second site the new section of artery is lengthening the original artery. much like inserting a new section of hose in a garden hose concentric with the longitudinal axis of the garden hose lengthens the garden hose."

This section clearly supports the examiner's interpretation, since the specification distinguishes between growth of a new artery adjacent to an existing artery and growing a new *section* of an artery integral with the original artery. The instant claims recite "forming a new artery" and not "forming a new *section of* an artery." Furthermore, it is noted that the claims were amended to recite "forming a *new* artery" in the amendment of 17 February 2004 so as to further distinguish the claimed invention from the disclosure of Murry et al. to overcome a rejection under 35 U.S.C. § 103(a) over Murry et al. (see p. 39 of the remarks section). Since Murry et al. disclosed growth of new capillaries, presumably by extension from (and integral with) existing arteries, Appellant's amendment was taken as distinguishing the claims from blood vessel

growth from existing arteries in an integral fashion. Considering all of the evidence, the interpretation of the claims is reasonable.

At p. 23 of the Appeal Brief of 24 October 2007, Appellant argues that the examiner has failed to identify any pre- or post-filing date art presenting evidence in regard to the subject matter of the claims on appeal which contradicts the objective evidence provided by the instant application. Appellant urges that the references cited by the examiner at p. 13 of the final rejection were only pertinent to claims which are now canceled. Finally, Appellant refers to Perin et al. as providing evidence of cardiac muscle growth and artery growth by providing "autopsy proof." This has been fully considered but is not found to be persuasive because it is factually incorrect. The rejection has referred to several publications for support of the position that undue experimentation, indeed, further acts of invention, were required by the skilled artisan to achieve repair of dead/damaged heart tissue by cell therapy. These include Strauer et al. 2002, Murry et al. 1996, Klug et al. 1996, Oakley et al. 2001, Chiu et al. 1995, Yoon et al. 1995, Koh, et al. 1993, Van Meter et al. 1995, and Koh et al. 1995, as explained in section 9 (Grounds of Rejection) above. No publications have been brought forth on the record to show that growth of a new artery can be achieved by cell therapy. This includes Perin et al. 2003. Appellant has previously provided an abbreviated epub advanced release of this article; however, a full copy of which has been scanned into the file for the convenience of the Board. Nowhere in the Perin et al. 2003 publication is reference made to an artery or arteriogenesis. No reference to an autopsy can be found. Perin et al. 2003 do refer to neovascularization, which is understood to involve

capillaries. Angiogenesis is also referred to by Perin et al. 2003, which encompasses growth of all types of blood vessels. It appears from the evidence appendix attached to the Appeal Brief of 24 October 2007 that it is possible that Appellant intended to refer to Dohmann et al. (2005, Circulation 112:521-526). Again, Appellant provided an epub document earlier in prosecution history. A full copy of the publication is provided for the convenience of the Board. Nowhere in Dohmann et al. 2005 is it stated that an artery was observed. The only conclusions made by Dohmann et al. were that the treated area had more capillaries than untreated areas (p. 526, conclusion section). Dohmann et al. state that some of the cells had acquired cytoskeletal elements and contractile proteins; however, Dohmann et al. carefully avoid concluding that arteries had been formed in this peer-reviewed publication. Therefore, the preponderance of the totality of the evidence indicates that growth of new arteries in dead/damaged heart tissue by cell therapy has not been achieved.

At pp. 24-25 of the Appeal Brief of 24 October 2007, Appellant presents a complicated argument, for the first time on this record, regarding the translation of dosages of nucleic acids as set forth in prophetic examples 18, 19, and 36 as guidance for dosages of cells. In this section, Appellant characterizes prophetic examples 18, 19, and 36 as providing guidance for dosages of cells and compares the calculated cell dosages to those used by Strauer et al. 2002. This has been fully considered but is not found to be persuasive. Prophetic examples 18, 19, and 36 *do not* provide any guidance whatsoever regarding dosages of *cells*. Rather, they provide suggestion regarding concentrations of cDNA plasmid molecules. The 6.25×10^6 and 12.5×10^6

figures do not ever appear in the instant specification, as alleged by Appellant. The suggested conversion formula presented in the footnote of p. 25 of the Appeal Brief of 24 October 2007 is simply nonsensical and scientifically unsound. No one of skill in the art would attempt to extrapolate a dosage for plasmid DNA to a dosage of cells containing a full genome, especially in the absence of any disclosure as to how to do so, or any disclosed suggestion to do so. Cells are not merely bags of DNA. Dosage considerations for naked DNA are different from those for cells. For example, DNA dosage calculations take into account degradation rates of DNA, and uptake rates of DNA into cells where they are expressed. Dosage calculations for cell therapy take into account, for example, survival and differentiation rates of the cells. Neither are considerations for the other, so a direct extrapolation based solely on a mathematical calculation is not scientifically sound. Prophetic examples 18, 19, and 36 simply do not suggest administration of cells for any purpose.

At p. 25-26 of the Appeal Brief of 24 October 2007, Appellant again states that the examiner has improperly performed a limited evaluation of the specification, and refers to a demonstration of such previously made in the arguments. This has been fully considered but is not found to be persuasive because one of the quotes was taken from a different rejection of different claims in a different application, as discussed above, and the other quote was partially directed to issues concerning the elected invention, and partially referred to issues concerning two words that no longer appear in any of the claims on appeal. Therefore, these sections of the office action are also not improper and irrelevant to the rejection being appealed.

At pp. 25-26 of the Appeal Brief of 24 October 2007, Appellant urges that the specification enables one skilled in the medical arts to make and use the invention. Appellant again refers to pp. 21, 45, 46, and examples 18, 19, and 36. At pp. 26-28 of the Appeal Brief of 24 October 2007, Appellant again argues that the materials and methods used in the claimed methods are old, and that such supports a finding of enablement. Appellant requests that the Board take "official notice" that processing bone marrow and peripheral blood for recovering mononuclear stem cells was routine in the medical arts prior to Appellant's invention. Appellant refers to cell banks and publications as evidence that stem cells from bone marrow were old in the art at the time of filing. Appellant concludes at p. 28 of the Appeal Brief of 24 October 2007 that, once the relevant materials and administration techniques set forth in the specification are properly considered in their entirety, there should be no question that one skilled in the medical arts is enabled to make and use the claimed invention. Appellant urges that the materials and administration techniques, but not the results, were well known at the filing date. This has been fully considered but is not found to be persuasive. Again, while the specification states that virtually any type of known "growth factor" as defined by the specification can be administered in any known way to grow virtually any desired body part, the specification does not provide guidance as to *how* such can be achieved. For example, the specification states that administration of a growth factor results in organogenesis, such that an artery or a heart or a pancreas or an eye or a tooth, etc., can be grown. How does the skilled artisan manipulate the conditions to achieve growth of an artery at the site of heart damage instead of growth of an eye, for

example? There is simply no explanation in the specification of how one skilled in the art can achieve the desired result at the desired site in the body.

At pp. 28-29 of the Appeal Brief of 24 October 2007, Appellant argues that the examiner has failed to comprehend that the specification discloses old and routine administration techniques and old materials to achieve a remarkable new result. Appellant urges that the skilled artisan would not need an extensive, detailed description of old elements of the invention and thus would be enabled to make and use the claimed invention once guided by the suggestions in the specification. Appellant argues that the examiner has provided erroneous assessments of Strauer et al. 2002, Deb et al., and Wollert et al. Appellant urges that a fair and reasonable reading of Strauer et al. 2002 and Deb et al., combined with the alleged evidence of Wollert et al. that high pressure technique is not necessary, leads to a conclusion of enablement. Appellant argues that the examiner's contention that the specification's lack of including Strauer et al. 2002's high pressure technique is nonenabling is erroneous. Appellant characterizes the examiner's conclusion as erroneous, not supported by sound, objective evidence, and speculative. This has been fully considered but is not found to be persuasive. As argued repeatedly above, the failure of the specification is in not providing guidance as to *how* old materials and methods can be used to achieve "remarkable" results. Appellant claims to have achieved something no one else had done simply by writing it down. To say that non-obvious and remarkable results can be achieved without doing a single experiment is incredible. It is a remarkable achievement to grow a new artery by implanting cells, but Appellant did not do it and Appellant's

disclosure does not teach anyone how to do it. Regarding Strauer et al. 2002, the examiner maintains that this publication constitutes evidence of the further act of invention that was required before achieving any repair of dead/damaged heart tissue. It is important to remember that even Strauer et al. 2002 did not find evidence of new artery growth, and thus even this advanced cell therapy method did not achieve the results required by the instant claims. Deb et al. was relied on by Appellant to show that cells injected at a site distant from the heart can migrate to the heart. However, Deb et al. do not demonstrate that cells can migrate to the heart in sufficient quantities to repair any defects. Deb et al. disclose that only 0.23 $\pm$ 0.06% of the cardiomyocytes were from the transplanted cells. Such numbers of cells are greatly insufficient to achieve the effects required by the claims. As evidence of this, Strauer et al. 2002 administered 6 to 7 fractional high-pressure infusions of 2 to 3 mL cell suspension, each of which contained 1.5 to 4 X 10^6 mononuclear cells directly to the infarct site in order to achieve their effects. In fact, Strauer et al. 2002 specifically points to shortcomings of intravenous administration such as that used by Deb et al. at p. 1917. The evidence as a whole indicates that intravenous administration of cells to repair a dead or damaged portion of a heart has not yet been achieved due to the obstacles involved with getting sufficient numbers of cells to the dead/damaged site and preventing them from re-migrating away from the site. As this problem has not yet been solved in the literature, and no suggestions for solving the problem are suggested in the specification as originally filed, undue experimentation would be required of the skilled artisan to practice the claimed method to achieve the required result. Finally, it is important to

note that Deb et al. do not demonstrate repair of dead/damaged heart tissue or growth of a new artery as required by the claims, despite disclosing a major method step, i.e., placing a cell in the body of a patient. Regarding Wollert et al., Wollert et al. is limited to multiple intraluminal injections into the infarct-related coronary artery, right at the site of the injured tissue. The specification does not provide guidance along the lines of Wollert et al.'s use of large quantities of cells and multiple administration passages to compensate for re-migration problems identified by others in this art. Furthermore, Wollert et al. do not disclose growth of a new artery, again constituting evidence that this has not been achieved in this art.

At pp. 29-30 of the Appeal Brief of 24 October 2007, Appellant argues that, even if the examiner had established a *prima facie* case of lack of enablement, that such has been rebutted by the multiple declarations of Drs. Heuser and Lorincz. Appellant urges that these two highly skilled medical experts read the relevant portions of the specification and reached the determination that one skilled in the medical arts, armed with the knowledge in the disclosures, would be enabled to practice the claimed methods and to predictably anticipate the results defined therein without resorting to undue experimentation. Appellant specifically refers to paragraphs 5-7 of the third supplemental declaration of Dr. Lorincz and paragraphs 5-7 of the fourth supplemental declaration of Dr. Heuser. This has been fully considered but is not found to be persuasive. The third supplemental declaration of Dr. Lorincz and the fourth supplemental declaration of Dr. Heuser under 37 CFR 1.132 filed 26 June 2006 are insufficient to overcome the rejection of claims 236, 238, 239, 243, 244, 247, 250, 251,

253, 257-263, 268-271, and 280-285 based upon 35 U.S.C., § 112, first paragraph, for lack of enablement as set forth in the last Office action for the following reasons. In assessing the weight to be given expert testimony, the examiner may properly consider, among other things, the nature of the fact sought to be established, the strength of any opposing evidence, the interest of the expert in the outcome of the case, and the presence or absence of factual support for the expert's opinion. See Ex parte Simpson, 61 USPQ2d 1009 (BPAI 2001), Cf. Redac Int'l. Ltd. v. Lotus Development Corp., 81 F.3d 1576, 38 USPQ2d 1665 (Fed. Cir. 1996), Paragon Podiatry Lab., Inc. v. KLM Lab., Inc., 948 F.2d 1182, 25 USPQ2d 1561, (Fed. Cir. 1993). In this case, the nature of the fact sought to be established is whether or not more than routine experimentation would have been required to practice the claimed invention in its full scope. This issue has been extensively addressed on the record with reference to the Wands factors and the publications of record. It is maintained that more than routine experimentation would have been required. The strength of opposing evidence has also been addressed extensively on the record. The post-filing date publications (such as Strauer et al. 2002) are filled with specific guidance necessary to achieve repair of dead/damaged heart tissue. This specific guidance is absent in the instant specification. Neither the specification nor any prior or post-filing date art has achieved growth of new arteries as required by the claims. Finally, the claims are incredibly broad, with the broadest claims reciting "placing" a growth factor at an undefined area of a human patient to form new cardiac muscle and a new artery in a pre-existing heart. The post-filing date art that achieves any positive effect on heart tissue used specific administration methods that

are not specifically pointed to in the specification, and no art of record achieves formation of a new artery. Thus, insofar as Appellant has brought forth Strauer et al. 2002 and other post-filing date publications to support enablement, it is noted that such evidence is not commensurate with the scope of the claims. Regarding the interest of the experts in the outcome of the case, there is no evidence that there is any such interest. Finally, there is a question of the presence or absence of factual support for the expert's opinion. It appears that the experts relied upon the specification itself, which has been separately addressed. Thus, the declarations have been fully considered and a finding that the rejection should be maintained is proper. As an aside, it is respectfully submitted that enablement is not a question of fact. Case law has established that anticipation and operativeness are questions of fact; however, obviousness and enablement are questions of law. See In re Lindell, 155 USPQ 521; In re Chilowsky, 134 USPQ 515. Thus, while no weight is given to the experts' opinion regarding the ultimate legal conclusion of enablement, the underlying basis for the legal conclusion has been considered and found to be insufficient to overcome the rejection.

At p. 30 of the Appeal Brief of 24 October 2007, Appellant argues that the examiner attempted to diminish the weight to be accorded to such declarations by essentially rearguing that more than routine experimentation would be required. Appellant urges that the examiner's arguments do not appear to be directly related to the claims on appeal. Appellant asserts that the examiner has not addressed the probative value of the objective evidence in the declarations. Appellant argues that the examiner, allegedly not a skilled person in the medical arts, has improperly relied solely

on her opinion. This has been fully considered but is not found to be persuasive because it is factually incorrect. The examiner has painstakingly considered the specification and all of the supplemental evidence of record in light of the Wands factors and reached a well-supported conclusion of non-enablement. Nowhere on the record has a statement been made along the lines of, "it is the examiner's opinion that the specification is not enabled."

At pp. 30-31 of the Appeal Brief of 24 October 2007, Appellant argues that the examiner has erroneously implied that the experts relied on "some publications" in addition to the disclosure. Appellant urges that a concise reading of the declarations reveals that the declarants relied solely upon the specification coupled with their skills in the medical arts. This has been fully considered but is not found to be persuasive. Several declarations by Drs. Heuser and Lorincz have been submitted as evidence. Some of these refer to publications at least in part. It is true that the third supplemental declaration of Dr. Lorincz and the fourth supplemental declaration of Dr. Heuser rely solely on the specification. As such, they do not add much to the record, since the specification has been thoroughly analyzed in view of the Wands factors. Again, it is noted that enablement is a question of law. No weight is given to the experts' opinion regarding the ultimate legal conclusion of enablement. See In re Lindell, 155 USPQ 521; In re Chilowsky, 134 USPQ 515.

At p. 31 of the Appeal Brief of 24 October 2007, Appellant argues a final point regarding the Wands factors. Specifically, Appellant argues that the instant fact pattern is similar to that in Wands because the skill level is high in the art and that known

materials and administration techniques were used. Appellant also again refers to the expert opinion declarations of Drs. Heuser and Lorincz. Appellant urges that proper consideration of all of these factors compels a conclusion that undue experimentation would not be required. This has been fully considered but is not found to be persuasive. The court in Wands stated that enablement is not precluded by the necessity for some experimentation such as routine screening. (*In re Wands*, 8 USPQ2d 1400 at p. 1404). In the instant case, however, what is missing is well beyond routine screening. The specification states that a growth factor can be placed in a patient and new cardiac muscle and a new artery will form and dead/damaged heart tissue will be repaired. The specification states that this *can* be done, but is does not state *how* it is to be done. How does one place a growth factor and achieve artery growth and not eye or kidney growth, as the specification states is also what *can* be done when placing a growth factor in the body of a patient? Furthermore, a very important distinction must be drawn between the fact patterns of the instant case and that of Wands. In Wands, evidence of multiple successful antibodies was provided. In the instant case, no evidence of growth of new cardiac muscle and a new artery in a heart has been brought forth on the record, or could be identified independently by the examiner.

The courts have stated that "[p]atent protection is granted in return for an enabling disclosure of an invention, not for vague intimations of general ideas that may or may not be workable". Genentech Inc. v. Novo Nordisk A/S (CAFC) 42 USPQ2d 1001 (1997). The courts have also stated that "[t]ossing out the mere germ of an idea does not constitute an enabling disclosure... [R]easonable detail must be provided in

order to enable members of the public to understand and carry out the invention" (Genentech Inc. v. Novo Nordisk A/S, *supra*). The instant specification provides only vague intimations of general ideas, or the mere germ of an idea along the lines of the specifications at issue in these pieces of case law.

In conclusion, a fresh consideration of the totality of the evidence of record shows that a preponderance of the totality of the evidence supports the rejection. Due to the large quantity of experimentation necessary to determine how to effectively administer cells to achieve *de* novo formation of cardiac muscle and an artery and thereby repair a dead/damaged portion of a heart, the lack of direction/guidance presented in the specification regarding how to achieve this remarkable result, the absence of working examples (real or prophetic) directed to the same, the complex nature of the invention, the contradictory state of the prior art (e.g., Strauer et al. 2002, Wollert et al.), the unpredictability of the effects of an agent on a physiological response, and the breadth of the claims which fail to recite limitations regarding cell type or site of delivery, etc., undue experimentation would be required of the skilled artisan to make and/or use the claimed invention in its full scope.

(11) Related Proceeding(s) Appendix

No decision rendered by a court or the Board is identified by the examiner in the Related Appeals and Interferences section of this examiner's answer.

(12) Oral Hearing

At this time, it does not appear that Appellant has requested an oral hearing. However, in the event that Appellant makes such a request, the examiner specifically requests the opportunity to present oral arguments at the Appeal hearing.

For the above reasons, it is believed that the rejections should be sustained.

Respectfully submitted,

/Elizabeth C. Kemmerer/

Primary Examiner, Art Unit 1646

Conferees

MANJUNATH RAO
SUPERVISORY PATENT EXAMINER

GARY B. NICKOL, PH.D.
SUPERVISORY PATENT EXAMINER
TECHNOLOGY CENTER 1600

Circulation
JOURNAL OF THE AMERICAN HEART ASSOCIATION

American Heart Association®
Learn and Live℠

Transendocardial, Autologous Bone Marrow Cell Transplantation for Severe, Chronic Ischemic Heart Failure

Emerson C. Perin, Hans F.R. Dohmann, Radovan Borojevic, Suzana A. Silva, Andre L.S. Sousa, Claudio T. Mesquita, Maria I.D. Rossi, Antonio C. Carvalho, Helio S. Dutra, Hans J.F. Dohmann, Guilherme V. Silva, Luciano Belém, Ricardo Vivacqua, Fernando O.D. Rangel, Roberto Esporcatte, Yong J. Geng, William K. Vaughn, Joao A.R. Assad, Evandro T. Mesquita and James T. Willerson

Circulation 2003;107;2294-2302; originally published online Apr 21, 2003;
DOI: 10.1161/01.CIR.0000070596.30552.8B
Circulation is published by the American Heart Association. 7272 Greenville Avenue, Dallas, TX 72514
 Print ISSN: 0009-7322. Online ISSN: 1524-4539

Transendocardial, Autologous Bone Marrow Cell Transplantation for Severe, Chronic Ischemic Heart Failure

Emerson C. Perin, MD, PhD*; Hans F.R. Dohmann, MD*; Radovan Borojevic, PhD; Suzana A. Silva, MD; Andre L.S. Sousa, MD; Claudio T. Mesquita, MD, PhD; Maria I.D. Rossi, PhD; Antonio C. Carvalho, MD, PhD; Helio S. Dutra, PhD; Hans J.F. Dohmann, MD, PhD; Guilherme V. Silva, MD; Luciano Belém, MD; Ricardo Vivacqua, MD; Fernando O.D. Rangel, MD; Roberto Esporcatte, MD; Yong J. Geng, MD, PhD; William K. Vaughn, PhD; Joao A.R. Assad, MD; Evandro T. Mesquita, MD, PhD; James T. Willerson, MD

Background—This study evaluated the hypothesis that transendocardial injections of autologous mononuclear bone marrow cells in patients with end-stage ischemic heart disease could safely promote neovascularization and improve perfusion and myocardial contractility.

Methods and Results—Twenty-one patients were enrolled in this prospective, nonrandomized, open-label study (first 14 patients, treatment; last 7 patients, control). Baseline evaluations included complete clinical and laboratory evaluations, exercise stress (ramp treadmill), 2D Doppler echocardiogram, single-photon emission computed tomography perfusion scan, and 24-hour Holter monitoring. Bone marrow mononuclear cells were harvested, isolated, washed, and resuspended in saline for injection by NOGA catheter (15 injections of 0.2 cc). Electromechanical mapping was used to identify viable myocardium (unipolar voltage ≥6.9 mV) for treatment. Treated and control patients underwent 2-month noninvasive follow-up, and treated patients alone underwent a 4-month invasive follow-up according to standard protocols and with the same procedures used as at baseline. Patient population demographics and exercise test variables did not differ significantly between the treatment and control groups; only serum creatinine and brain natriuretic peptide levels varied in laboratory evaluations at follow-up, being relatively higher in control patients. At 2 months, there was a significant reduction in total reversible defect and improvement in global left ventricular function within the treatment group and between the treatment and control groups ($P=0.02$) on quantitative single-photon emission computed tomography analysis. At 4 months, there was improvement in ejection fraction from a baseline of 20% to 29% ($P=0.003$) and a reduction in end-systolic volume ($P=0.03$) in the treated patients. Electromechanical mapping revealed significant mechanical improvement of the injected segments ($P<0.0005$) at 4 months after treatment.

Conclusions—Thus, the present study demonstrates the relative safety of intramyocardial injections of bone marrow–derived stem cells in humans with severe heart failure and the potential for improving myocardial blood flow with associated enhancement of regional and global left ventricular function. **(*Circulation*. 2003;107:2294-2302.)**

Key Words: cells ■ heart failure ■ ischemia ■ revascularization ■ gene therapy

After myocardial infarction, chronically ischemic (hibernating) myocardium may persist in association with variable degrees of scar tissue. In most circumstances, native angiogenesis is insufficient to prevent the resultant remodeling when significant injury occurs. As a consequence, infarct-related heart failure remains a major cause of morbidity and mortality.

The understanding that vasculogenesis can occur in the adult has led to intense investigation into stem cell therapy. Several recent experimental studies have confirmed the potential of pluripotential cells in differentiating into cardiomyocytes and endothelial cells.[1,2] Further evidence from animal models has confirmed that pluripotential cells from

Received March 7, 2003; revision received March 25, 2003; accepted March 26, 2003.

From the Texas Heart Institute at St Luke's Episcopal Hospital, Houston, Tex (E.C.P., G.V.S., Y.J.G., W.K.V., J.T.W.); Hospital Procardiaco, Rio de Janeiro, Brazil (H.F.R.D., S.A.S., A.L.S.S., C.T.M., H.J.F.D., L.B., R.V., F.O.D.R., R.E., J.A.R.A., E.T.M.); Federal University, Rio de Janeiro, Brazil (R.B., M.I.D.R., A.C.C., H.S.D.); and Brazilian Millennium Institute for Tissue Bioengineering (H.F.R.D., R.B., A.C.C.).

*Drs Perin and Dohmann are co-principal investigators.

Guest editor for this article was Valentin Fuster, MD, PhD, Mount Sinai School of Medicine, NY.

This article originally appeared Online on April 21, 2003 (*Circulation*. 2003;107:r75–r83).

Correspondence to Emerson C. Perin, MD, 6624 Fannin, Suite 2220, Houston, TX 77030 (e-mail eperin@crescentb.net), and Hans F.R. Dohmann, MD, Rua General Polidoro, 192, CEP 22080-000–Botafogo, Rio de Janeiro, Brazil (e-mail hemodinamica@procardiaco.com.br).

Circulation **is available at http://www.circulationaha.org** **DOI: 10.1161/01.CIR.0000070596.30552.8B**

bone marrow improve myocardial function and perfusion in the setting of ischemic heart disease.[3,4] In addition, recent publications[5,6] have described beneficial effects of intracoronary infusion of autologous, mononuclear bone marrow in the immediate postinfarction period in humans. A recent report by Tse et al[7] described improvement in myocardial perfusion and segmental contractility (as assessed by cardiac magnetic resonance imaging) in ischemic myocardial segments treated with catheter-based delivery.

The present study addresses primarily the safety of endocardial bone marrow mononuclear cell (BMMNC) injections and secondarily the hypothesis that endocardial injections of autologous BMMNCs (ABMMNCs) in patients with end-stage ischemic heart disease may promote neovascularization and may overcome the failure of the natural myocardial healing process.

Methods

Patient Population

This is a prospective, nonrandomized, open-label study of 21 patients with severe ischemic heart failure and no other option for standard revascularization therapies. Patients were enrolled sequentially, with the first 14 patients assigned to the treatment group and the last 7 patients to the control group. In accordance with the ethics committee's recommendations, an initial group of 4 patients was enrolled as a safety study. After 4 months' follow-up of the initially injected patients (once safety was determined), the remaining study patients were enrolled. All patients were placed on maximally tolerated medical therapy at time of enrollment. The following inclusion criteria were required for patient enrollment: (1) chronic coronary artery disease with reversible perfusion defect detectable by single-photon emission computed tomography (SPECT); (2) left ventricular (LV) ejection fraction (EF) <40%; (3) ineligibility for percutaneous or surgical revascularization, as assessed by coronary arteriography; and (4) signed, informed consent. Incligibility for surgical or percutaneous revascularization procedures was determined by 2 expert committees: a surgical committee comprising 2 cardiovascular surgeons and a noninvasive cardiologist, and an interventional committee comprising 2 interventional cardiologists and 1 noninvasive cardiologist. Patients were not enrolled in the study if any 1 of the following exclusion criteria was met: (1) difficulty in obtaining vascular access for percutaneous procedures; (2) previous or current history of neoplasia or other comorbidity that could impact the patient's short-term survival; (3) significant ventricular dysrhythmias (sustained ventricular tachycardia); (4) LV aneurysm; (5) unexplained abnormal baseline laboratory abnormalities; (6) bone tissue with abnormal radiological aspect; (7) primary hematologic disease; (8) acute myocardial infarction within 3 months of enrollment in the study; (9) presence of intraventricular thrombus by 2D Doppler echocardiogram; (10) hemodynamic instability at the time of the procedure; (11) atrial fibrillation; or (12) any condition that, in the judgment of the investigator, would place the patient at undue risk.

The ethics committee of Pro-Cardiaco Hospital (Rio de Janciro) and the Brazilian National Research Ethics Council approved the study protocol.

Baseline Evaluation

Baseline evaluation in the treatment group included a complete clinical evaluation (history and physical), laboratory evaluation (complete blood count, blood chemistry, C-reactive protein [CRP], brain natriuretic peptide [BNP], creatine kinase [CK]-MB and troponin serum levels), exercise stress test with ramp treadmill protocol,[8] 2D Doppler echocardiogram, dipyridamole SPECT perfusion scan, and 24-hour Holter monitoring.

The control group underwent the above-mentioned baseline evaluation except for 24-hour Holter monitoring, CK-MB, and troponin serum levels.

Periprocedural Evaluation

Patients in the treatment group had serum CRP, complete blood count, CK, troponin, and BNP (only 9 patients) levels measured and an ECG performed just before the procedure. Immediately after the procedure, another ECG and 2D Doppler echocardiogram were performed, and 24-hour Holter monitoring was begun. Serum CRP, CK, and troponin levels were also assessed at 24 hours. Patients were monitored in the cardiac intensive care unit for 48 hours after the injection procedure.

Bone Marrow Aspiration and Isolation of Mononuclear Cells

Approximately 4 hours before the cell injection procedure, bone marrow (50 mL) was aspirated under local anesthesia from the posterior iliac crest. BMMNCs were isolated by density gradient on Ficoll-Paque Plus (Amersham Biosciences). Mononuclear cells were exhaustively washed with heparinized saline containing 5% human serum albumin and filtered through 100-μm nylon mesh to remove cell aggregates. The cells were finally resuspended in saline with 5% human serum albumin for injection. A small fraction of the cell suspension was used for cell counting and viability testing with trypan blue exclusion. Cell viability was shown to be >90% (96.2±4.9%), assuring the quality of the cell suspension. Post-hoc characterization of leukocyte differentiation markers by flow cytometry and functional assays was done on another fraction of cells. The clonogenic capacity of hematopoietic progenitors was evaluated by colony-forming assays (granulocyte-macrophage colony-forming unit) as previously described.[9]

A high correlation between granulocyte-macrophage colony-forming units and $CD45^{lo}CD34^{+}$ cells was seen (Spearman $r=0.77$, $P=0.0012$). Fibroblast colony-forming assay was done as previously described[10] to determine the presence of putative progenitor mesenchymal lineages. Bacterial and fungal cultures of the clinically used cell preparations were performed and proved negative.

Antibodies and Staining Procedure for Fluorescence-Activated Cell Sorter Analysis

The following antibodies were either biotinylated or conjugated with fluorescein isothiocyanate (Pharmingen), phycoerythrin (PE), or PerCP: anti-CD45 as a pan-leukocyte marker (clone HI30), anti-CD34 as a hematopoietic progenitor marker (clone HPCA-II), anti-CD3 as a pan–T-cell marker (clone SK7), anti-CD4 as a T-cell subpopulation marker (clone SK3), and anti-CD8 as a T-cell subpopulation marker (clone SK1) from Becton Dickinson; anti-CD14 as a monocyte marker (clone TUK4), anti-CD19 as a pan–B-cell marker (clone SJ25-C1), and anti-CD56 as an NK-cell marker (clone NKI nbl-1), from Caltag Laboratories (Burlingame, Calif); and anti-HLA–DR (MHC-II, clone B8.12.2) from Beckman-Coulter. The biotinylated antibodies were revealed with Streptavidin PECy7 (Caltag Laboratories). Three-color immunofluorescence analysis was used for the identification of leukocyte populations in total nucleated bone marrow cell suspensions. After staining, erythrocytes were lysed with the Becton Dickinson lysis buffer solution according to the manufacturer's instructions, and CD45 antibody was used to assess the percentages of leukocytes in each sample. Data acquisition and analyses were performed on a fluorescence-activated cell sorter Calibur with CellQuest 3.1 software (Becton Dickinson).

Transendocardial Delivery of ABMMNCs

In the cell-injection treatment group, patients were taken to the cardiac catheterization laboratory ≈1 hour before the anticipated arrival of the bone marrow cells from the laboratory. Left heart catheterization with biplane LV angiography was performed. Subsequently, electromechanical mapping (EMM) of the left ventricle was performed as previously described.[11] The general region for treatment was selected by matching the area identified as ischemic

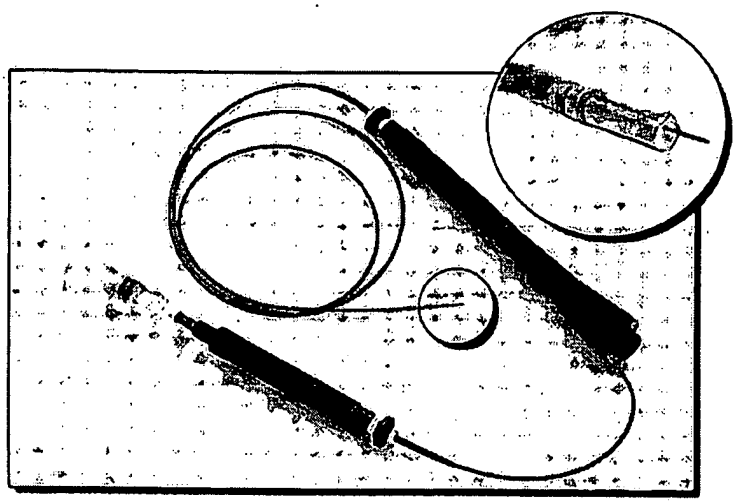


Figure 1. The NOGA Myostar injection catheter, with the needle in the extended position (insert).

by previous SPECT perfusion imaging. The electromechanical map was then used to target the specific treatment area by identifying viable myocardium (unipolar voltage ≥6.9 mV)[12] within that region. Areas associated with decreased mechanical activity (local linear shortening <12%, indicating hibernating myocardium) were preferred.

The NOGA injection catheter (Figure 1) was prepared by adjusting the needle extension at 0° and 90° flex and by placing 0.1 cc of ABMMNCs to fill the needle dead space. The injection catheter tip was placed across the aortic valve and into the target area, and each injection site was carefully evaluated before the cells were injected. Before every injection of cells into the LV wall, the following criteria had to be met: (1) perpendicular position of the catheter to the LV wall; (2) excellent loop stability (<4 mm); (3) underlying voltage >6.9 mV; and (4) presence of a premature ventricular contraction on extension of the needle into the myocardium. Fifteen injections of 0.2 cc (mean of $25.5\pm6.3\times10^6$ cells/patient) were delivered (Figure 2).

Two-Month Noninvasive Follow-Up Evaluation

All patients, both treated and control, underwent noninvasive follow-up evaluations at 2 months, which consisted of a clinical evaluation, ramp treadmill protocol, 2D Doppler echocardiogram, and dipyridamole SPECT perfusion scan. Patients in the treatment group had repeat 24-hour Holter monitoring. The ramp treadmill protocol was selected because it is better than standard incremental protocols in estimating functional capacity in these severely ill patients.[8]

The predicted $\dot{V}O_2$max was used to tailor the patient workload. Treadmill speed was initially 0.5 mph, and inclination was 0% to 10% with a planned duration of 10 minutes of exercise.[13,14] The echocardiographic data were analyzed by 2 independent, blinded, experienced observers. Images were stored digitally and analyzed offline. If a discrepancy between the readings of >5% was noted, a third blinded observer was called and a consensus achieved. The end-systolic volume (ESV), end-diastolic volume (EDV), and EF were measured according to standard protocols.

Dipyridamole stress and resting SPECT imaging were performed with the same stress procedure at baseline and at follow-up. Studies were read by a blinded, experienced observer. Approximately 740 MBq of technetium-99m sestamibi was injected at rest and after stress, with dipyridamole infusion at a rate of 142 μg/kg of body weight per minute infused for 4 minutes. One hour later, SPECT imaging was initiated, using a 15% window centered over the 140-keV photopeak. Acquisitions were performed with a 1-detector gamma camera (Ecam, Siemens), acquiring 32 projections over 180° (right anterior oblique 45° to left posterior oblique 45°) (low-energy, high-resolution collimation; 64×64 matrixes; and 35 seconds per projection). Short-axis and vertical and horizontal long-axis tomograms of the left ventricle were extracted from the reconstructed transaxial tomograms by performing coordinate transformation with appropriate interpolation. No attenuation or scatter correction was applied. Quantitative SPECT analysis was performed on an ICON workstation computer (Siemens). The analysis was performed with the use of a completely automated software package, with the exception of a quality-control check to verify the maximum count circumferential profiles. The methods for quantitative analysis have been previously described.[15,16] In brief, processing parameters, including the apical and most basal tomographic short-axis slices, the central axis of the LV chamber, and a limiting radius for myocardial count search, were automatically derived. Short-axis tomograms

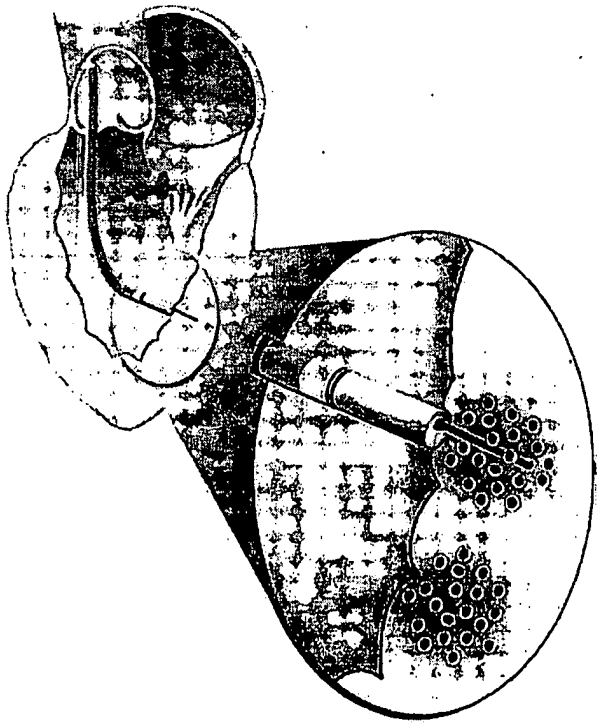


Figure 2. Injection catheter advanced into the left ventricle through the aortic valve. The catheter tip is placed against the endocardial surface (insert) with the needle extended into the myocardium delivering ABMMNCs.

TABLE 1. Demographics of the Treatment and Control Groups

	Treatment (n=14)	Control (n=7)	P
Age	56.9±9.8	64.3±7.2	0.1
Male gender, %	86	90	0.53
Hypertension, %	64	71	0.74
Diabetes, %	29	57	0.35
Hypercholesterolemia, %	79	57	0.35
Smoking, %	7	0	0.47
Previous myocardial infarction, %	100	100	1.0
Previous percutaneous coronary intervention, %	7	43	0.09
Previous coronary artery bypass grafting, %	64	86	0.61
Previous stroke, %	29	0	0.26
Peripheral vascular disease, %	57	71	0.66
Chronic renal failure, %	14	14	1.0
Multivessel disease, %	100	100	1.0

Values are mean±SD or percentage of patients.

were then sampled by using a maximum-count circumferential profile sampling technique with a cylindrical approach for sampling the body of the left ventricle and a spherical approach for sampling the LV apex. Comparisons were made to sex-matched normal limits.[16] Polar map displays and quantitative values were then generated to indicate stress myocardial perfusion defect extent and severity.[16,17]

Four-Month Invasive Follow-Up Evaluation

Patients in the control group did not undergo NOGA mapping or repeat LV angiograms at late follow-up (because of ethics committee recommendations).

Patients in the treatment group had 4-month invasive follow-up evaluations consisting of LV angiograms and EMM. LV angiography was performed through the femoral approach with the use of a 5F pigtail catheter. All angiograms were obtained in 2 planes—a 30° right anterior oblique view and a 60° left anterior oblique view—during a period of stable sinus rhythm. Ventricular volume was not measured during or after a premature beat. A 40-mm sphere was used as calibration device. LV EDV, ESV, and EF were calculated by 2 blinded, experienced observers who used the area-length method.[18]

EMM was performed according to established criteria[11] with a fill threshold of 15 mm. After the acquisition of points, postprocessing analysis was performed with a series of filters (moderate setting) to eliminate inner points, points that do not fit the standard stability criteria (location stability <4 mm, loop stability <6 mm, and cycle length variation <10%), points acquired during ST-segment elevation, and points not related to the left ventricle (eg, those in the atrium). A blinded, expert observer used a 12-segment bull's-eye to compare electromechanical values (unipolar voltage and local linear shortening) of injected segments at baseline and follow-up.

Statistical Analyses

Univariate differences in demographic characteristics (Table 1) between the control and treated groups were assessed with χ^2/Fisher's exact test and t tests for discrete and continuous variables, respectively. Multivariable logistic regression was also used to determine the independent relationship between each demographic variable and treatment group. No statistically significant differences between the 2 groups were found. Because each patient in both groups was used as his or her own control, changes between baseline and 8 weeks in the control and treated groups were assessed with paired t tests. Logistic regression analysis was utilized to compare medications (Table 2) at baseline, 8 weeks, and 16 weeks within the control and treatment groups and between the control and treatment groups.

TABLE 2. Percentage of Patients Receiving Selected Cardiac Medications at Baseline and 8- and 16-Week Follow-Up

	Baseline	8 Weeks	16 Weeks	P
ACE+ARB				
Control	86	86	86	1.0
Treatment	86	100	93	0.32
P	0.63*			
Nitrates				
Control	86	86	86	0.99
Treatment	93	93	93	0.91
P	0.95*			
β-Blockers				
Control	43	57	57	0.59
Treatment	71	71	64	0.73
P	0.94*			
Diuretics				
Control	71	71	71	1.0
Treatment	86	79	71	0.56
P	0.65*			
Ca channel blockers				
Control	14	14	29	0.49
Treatment	21	29	21	0.89
P	0.62*			

*P for comparison of all 3 time periods between treatment and control groups.

Comparisons of the changes from baseline to 8 weeks in the control and treatment groups were made with repeated-measures ANOVA. The ANOVA model included the control versus treatment and baseline versus 8 weeks as factors and also included the interaction between the 2 factors. A probability value <0.05 was considered statistically significant.

Results

Patient population demographics did not differ significantly between the treatment and control groups (Table 1). There were no significant differences in β-blocker, ACE inhibitor, or nitrate use between the 2 groups (Table 2).

Procedural Data

The total procedural time for mapping and injection was 81±19 minutes. Electromechanical maps comprised an average of 92±16 points. Patients received an average of 15±2 cell injections in a mean of 2±0.7 segments (6 inferior, 14 lateral, 2 anterior, and 5 septal). Each injection of 2 million cells was delivered in a volume of 0.2 cc. The cell population comprised a mean of 2.44±1.33% $CD45^{lo}CD34^{+}$ cells (Table 3).

Safety Data

One patient in the control group died 2 weeks after enrollment in the study and was not included in the analysis. A patient in the treatment group died at 14 weeks, presumably of sudden cardiac death. This patient had onset of severe angina and was found to be in asystole by emergency medical personnel. The patient had persistent improvement in cardiac

TABLE 3. Characteristics of Bone Marrow Mononuclear Cells Injected Into the Myocardium*

Cell Population and Phenotype	Percent of Injected Cells	No. of Cells Injected, ($\times 10^3$)/mm^2
Hematopoietic progenitor cells ($CD45^{lo}CD34^+$)	2.4±1.3*	57.4±61.4*
Early hematopoietic progenitor cells ($CD45^{lo}CD34^+HLA\text{-}DR^-$)	0.1±0.1	2.1±1.8
$CD4^+$ T cells ($CD45^+CD3^+CD4^+$)	28.4±10.8	537.0±265.7
$CD8^+$ T cells ($CD45^+CD3^+CD8^+$)	14.9±5.9	311.0±221.6
B cells ($CD45^+CD19^+$)	1.9±1.0	232.5±174.8
Monocytes ($CD45^+CD14^+$)	10.0±4.0	202.8±161.0
NK cells ($CD45^+CD56^+$)	1.2±0.5	21.2±13.5
Functional assay	**No. Colonies/10^6 BMMNC**	**No. of Cells Injected, ($\times 10^3$)/mm^2**
Fibroblast colony-forming assay	7.8±9.7	0.2±0.2
Granulocyte-macrophage colony-forming unit assay	719.6±385.3	16.4±18.5

Values are average±SD.

*Results for 14 patients in the treatment group, except: $CD34^+CD45^{lo}HLA\text{-}DR^-$, 13 patients; $CD45^+CD19^+$, 13 patients; $CD45^+CD14^+$, 11 patients; and $CD45^+CD56^+$, 9 patients.

function, as assessed by echocardiography. Baseline EF was 30% by echocardiography and increased to 57% at 2-month follow-up, demonstrating a similar response as the rest of the treatment group with regard to increased contractile function. In both cases, the families refused postmortem exams.

There were no major periprocedural complications. One patient had a transient episode of pulmonary edema that was easily reversed with loop diuretics after the procedure. No sustained arrhythmias were associated with the injection procedures, nor did any significant arrhythmias occur while the patients were hospitalized. There were no sustained ventricular arrhythmias found on 24-hour Holter monitoring at baseline or when repeated after the injection procedure and no significant differences in the number or percentage of premature ventricular contractions. No postprocedural pericardial effusions were seen on 2D Doppler echocardiograms. All patients were discharged on the third hospital day as per protocol.

Two-Month Noninvasive Follow-Up Evaluations

Of all baseline and follow-up laboratory values (Table 4), only serum creatinine and BNP levels varied between the control and treatment groups at follow-up. Follow-up serum creatinine levels were significantly elevated in the control group as compared with the treatment group ($P=0.03$). The levels of CRP at baseline and follow-up were not significantly different between the two groups (Table 4). There was a trend toward increased difference of BNP levels at follow-up between the two groups, with higher levels in the control group ($P=0.06$).

Patients in the treatment group experienced less heart failure and fewer anginal symptoms at the 2-month follow-up when compared with the control group, by both New York Heart Association (NYHA) and Canadian Cardiovascular Society Angina Score (CCSAS) distribution (Table 5). Baseline exercise test variables (METs and $\dot{V}O_2$max) were similar for the 2 groups. There was a significant increase, however, in METs and $\dot{V}O_2$max at follow-up in the treatment group ($P=0.0085$ and 0.01, respectively). There was a trend toward improvement when these variables were compared with the control group ($P=0.08$ for both variables).

Baseline comparison of ESV, EDV, and LVEF between the treatment and control groups revealed significant differ-

TABLE 4. Laboratory Values for the Treatment and the Control Groups

	Treatment (n=14)	Control (n=7)	*P*
White blood cells, nL			
Before treatment	8.3±2.8	8.6±1.5	0.39
After treatment	8.3±2.1	9.2±1.4	0.19
P	0.85	0.14	
Creatinine, mg/dL			
Before treatment	1.17±0.32	1.35±1.02	0.60
After treatment*	1.10±0.26	1.63±0.08	0.030
P	0.23	0.09	
CRP, mg/dL			
Before treatment	1.00±0.70	0.76±0.50	0.43
After treatment	1.03±1.0	0.61±0.57	0.33
P	0.94	0.59	
BNP, pg/mL			
Before treatment	328.1±410.7	404.4±421.6	0.73
After treatment	281.8±286.6	565.1±366.3	0.06
P	0.91	0.19	
CK-MB, ng/mL			
Before treatment	2.67±0.42	NA	NA
24 Hours	3.08±1.43	NA	NA
P	0.35	NA	NA
Troponin, ng/mL			
Before treatment	0.14±0.09	NA	NA
24 Hours	1.13±0.84	NA	NA
P	0.0007	NA	NA

CK-MB indicates myocardial muscle creatine kinase isoenzyme; NA, not applicable.

*After treatment=2 months.

TABLE 5. Comparison of Baseline and 2-Month Follow-Up Values for the Treatment and Control Groups

	Treatment (n=14)	Control (n=7)	P*
NYHA class			
Before treatment	2.21±0.89	2.71±0.75	
After treatment	1.14±0.36	2.71±0.76	0.0001
P	0.0003	1.0	
CCSAS class			
Before treatment	2.64±0.84	2.57±0.97	
After treatment	1.28±0.61	2.14±0.89	
P	0.0001	0.06	0.001
Ramp treadmill			
METs			
Before treatment	5.09±2.5	5.07±1.96	
After treatment	6.68±2.35	5.16±2.45	0.078
P	0.0085	0.84	
Vo_2max			
Before treatment	17.96±8.78	17.75±6.85	
After treatment	23.38±8.31	18.08±8.58	0.08
P	0.01	0.84	
Echocardiogram			
ESV, cc			
Before treatment	146.78±53.46	89.42±26.23	
After treatment	123.21±47.88	98.85±20.52	0.041
P	0.026	0.36	
EDV, cc			
Before treatment	211.35±76.89	135.71±26.08	
After treatment	189.14±67.54	145±27.62	0.09
P	0.065	0.50	
EF, %			
Before treatment	30±5.56	36±11.73	
After treatment	35.5±7.85	31.85±7.55	0.029
P	0.027	0.31	
SPECT			
Total reversible defect, %			
Before treatment	15.15±14.99	10.71±16.60	
After treatment	4.53±10.61	32.28±37.25	0.022
P	0.016	0.23	
% Rest defect (50%)			
Before treatment	40.77±11.13	35.85±10.09	
After treatment	38.84±8.79	36.42±12.08	0.65
P	0.44	0.77	

*P values reflect comparison of the differences between treatment and control groups over time (see Methods).

ences: The control group had smaller LV volumes ($P<0.001$) and a trend ($P=0.054$) toward higher baseline EF. Cardiac function (measured by EF on echocardiograms) had an absolute increase of 6% over the 2-month follow-up period in the cell-treated group. In contrast, the mean EF decreased, although not significantly, in the control group. In addition, when the 2 groups were compared, the treatment group showed a significant improvement in EF after 2 months ($P=0.03$). Cardiac geometry, as assessed by ESV, also improved. A significant fall in ESV ($P=0.03$) and a trend toward reduction in EDV ($P=0.07$) were noted in the treatment group. Volumes remained unchanged within the control group. When the two groups were compared at follow-up, a significant reduction in ESV was seen in the treated patients ($P=0.04$).

Nuclear perfusion imaging studies were similar at baseline for the amount of total reversible defect and percent of rest defect with 50% activity (scar). Within the control group, there was no significant change in these two variables at follow-up. Within the treatment group, there was no significant change in rest defect, with 50% activity at 2-month follow-up, but there was a significant 73% reduction in total reversible defect ($P=0.022$; from 15.15±14.99% to 4.53±10.61%). A typical example of resolution of inferolateral ischemia (baseline to follow-up) in a cell-treated patient is shown in Figure 3A.

Four-Month Invasive Follow-Up Evaluations

Results from LV angiography at baseline and 4-month follow-up are shown in Table 6. There was a sustained improvement in LVEF from baseline, an increase from 20% to 29% at 4 months (31% relative increase) ($P=0.0003$) in the treated patients. There was also a continued reduction in ESV ($P=0.03$) at 4 months. EDV remained unchanged ($P=0.1$). Control group patients did not have repeat LV angiograms.

On EMM, segmental analysis revealed a significant mechanical improvement of the injected segments ($P<0.0005$) (Table 6). Significant improvement in mechanical function at the injection site is illustrated by EMM in Figure 3B. Unipolar voltage values did not change from baseline to follow-up.

Discussion

The present study describes for the first time ABMMNC transplantation with the use of transendocardial injections in patients with severe LV dysfunction, end-stage ischemic heart disease, and no other option for treatment. The results of our study suggest that injection of ABMMNCs is safe and improves perfusion and myocardial contractility when viable areas of myocardium are targeted.

Wound healing is a multifaceted process that involves complex interactions between inflammatory cells, cytokines, and a number of extracellular matrix proteins, and the development of new capillaries. Because the normal reparative mechanisms seem to be overwhelmed when clinically significant myocardial injury occurs, a logical next step would be to amplify one part of this response artificially by applying stem cells locally in the setting of ischemia or infarction when a large amount of heart muscle has been injured.

In experimental animals, bone marrow–derived cells have been shown to regenerate areas of infarcted myocardium and coronary capillaries,[1] thus limiting functional impairment after myocardial infarction. Transendocardial injection of ABMMNCs has been shown to increase myocardial contractility and perfusion in swine.[4] Various cell lineages have been used to generate evidence that bone marrow stem cells

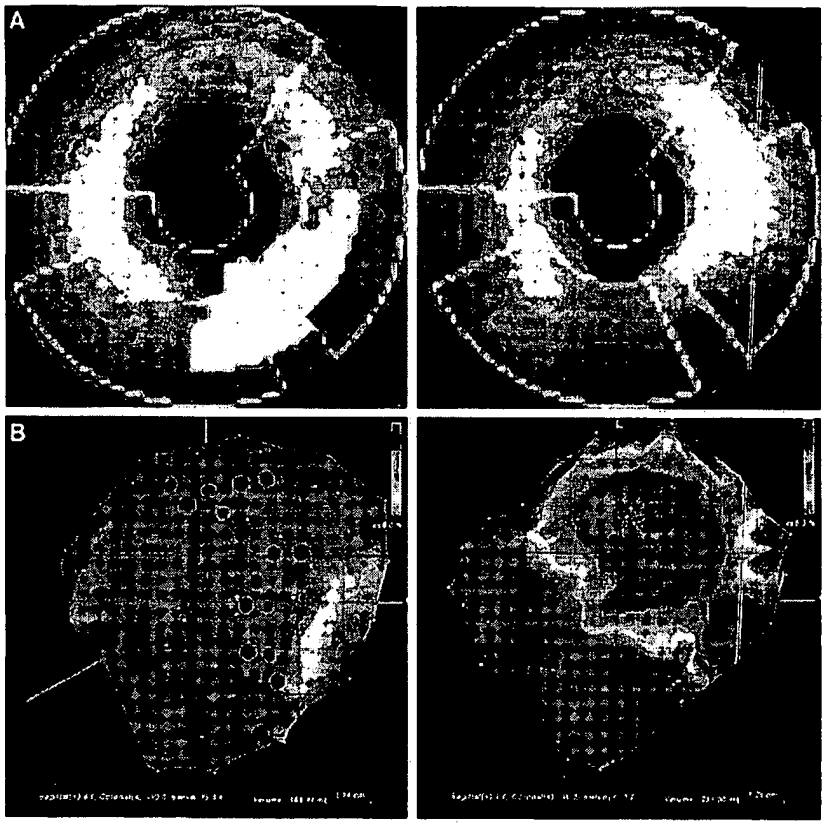


Figure 3. A, SPECT polar map at baseline, showing an area of inferolateral, reversible ischemia in white and nonreversible stress defect in black (left). Follow-up SPECT at 2 months, showing complete resolution of ischemic defect and basilar nonreversible defect with a decrease in nonreversible apical defect (right). B, Electromechanical maps from the same patient viewed from the inferior position. Mechanical map at the time of the injection procedure (left) shows the 15 injection sites in black distributed along the inferior wall. The follow-up mechanical map at 4 months (right) shows marked improvement in contractile function in the injected area.

differentiate into cardiomyocytes, endothelium, and smooth muscle cells.[19] Bone marrow hemangioblasts add to the development of new vessels, and mesenchymal stem cells can transdifferentiate into functional cardiomyocytes.[20] Recently, bone marrow–derived cardiomyocytes were demonstrated in hearts of women who received gender-mismatched bone marrow transplantation.[21] Moreover, bone marrow cellular components secrete a range of cytokines, fibroblast growth factor, and vascular endothelial growth factor,[22] which are involved in the natural process of angiogenesis. Endothelial progenitor cells have been implicated in neovascularization associated with postnatal vasculogenesis and are mobilized to peripheral circulation after acute ischemic events.[23]

In the present study, there is preliminary evidence that in humans, bone marrow–derived mononuclear cells are capable of enhancing perfusion, as shown by significant reductions in reversible stress defects on SPECT ($P=0.02$). Bone marrow–derived cells were purposefully injected into areas of hibernating myocardium. In hibernating areas, the underlying physiological state allows for restoration of myocardial function if myocardial perfusion is improved. We hypothesize that angiogenesis is the mechanism that allowed improvement in myocardial function in the patients in our study. Furthermore, we may speculate that an orchestrated sequence of events that includes not only the presence of the transplanted cells but also the action of cytokines and growth factors and intricate cell-to-cell interactions may all contribute to angiogenesis as an end result. Therefore, the resultant localized increase in contractility at cell injection sites, as seen by a significant increase in mechanical function on EMM, likely occurred as a consequence of an underlying improvement in perfusion. However, we cannot exclude the possibility that the injections themselves stimulated new blood vessel growth and enhanced function through the induction of angiogenic and important growth factors.

TABLE 6. Angiographic and EMM Results for the Treatment Group at 4 Months' Follow-Up (n=13)

	Before Treatment	After Treatment	*P*
LV angiogram			
EDV, cc	213.5±81.6	181±51.3	0.1
ESV, cc	174.1±78.7	133.5±54	0.03
EF, %	20±9	29±13	0.0003
EMM			
Unipolar voltage, mV	10.5±3.5	10.3±2.7	0.65
Local linear shortening, %	5.7±3.7	10.8±7.5	0.0005

The homing process, which results in cell engraftment, may also play a key role in the success of cell therapy. After acute events, serum vascular endothelial growth factor levels rise significantly,[23] and it is likely that homing signals may be more intense in acute and subacute ischemic syndromes. In our patients, all of whom had chronic disease, we opted to perform transendocardial cell-therapy delivery because we believe that homing signaling may not be as intense and, therefore, might not be optimal for cell engraftment. It is also likely that a smaller number of cells is required to achieve the desired effect.

EMM technology has been widely confirmed to be accurate for delineating and identifying scarred and viable myocardium and for differentiating degrees of infarct transmurality.[11,12,24] EMM thus offers a theoretical benefit over surgical or intracoronary approaches because viability of the site can be determined before each injection. Injections would then be performed only to targeted, viable areas of hibernating myocardium. Many treated sites targeted in this study were in areas of totally occluded epicardial vascular beds, making intracoronary delivery impossible. Furthermore, potential ischemia provoked by coronary manipulation is avoided. This approved procedure seemed safer for these chronically ill, high-risk patients because it avoided associated surgical morbidity and mortality.

Tse et al[7] recently demonstrated improvement in myocardial perfusion and segmental contractility after ABMMNC transendocardial injections. Those results are somewhat similar to results of the present study, although Tse and colleagues did not see improvement in global EF. The main difference between the studies is the significant baseline LV dysfunction present in our group (mean EF, 20%) as compared with a normal mean EF (56.9%) in the Tse study.[7] The preliminary data of Tse and colleagues also suggest the relative safety of the procedure.

The use of transendocardial delivery proved to be safe in our study, as cellular therapy was successfully delivered in every case without any major periprocedural events (eg, death, myocardial infarction, ventricular arrhythmias, cardiac perforation, pericardial effusion, or development of intramyocardial tumor). Troponin levels increased by a small but significant amount, consistent with delivery via intramuscular injection (Table 4), but the absolute rise was relatively small biologically. The stability between levels of CRP in the treatment and control groups suggests that we did not initiate a significant inflammatory reaction with cell injection.

The major limitations of this study are the small number of patients enrolled and the study design, which limits conclusions about efficacy. Because of ethics committee concerns, the control group was not enrolled concurrently with treated patients, did not receive a placebo injection, and did not undergo invasive follow-up. However, treatment and control groups had similar follow-up up to 2 months. The benefits seen in this study with cell therapy could be attributable to the placebo effect seen in phase I trials. Potential biases include selection bias (eg, tertiary hospital population) and investigator bias when assessing symptoms at follow-up (CCSAS and NYHA class) although echocardiographic, angiographic, and SPECT studies were read blindly. In addition, smaller LV volumes and a trend toward higher EFs were present in the control group. However, both groups were matched in terms of demographics, medication use, baseline laboratory values, functional status classification, treadmill workload, and Vo_2max. More importantly, similar baseline reversible and fixed ischemic defects were present in both groups, as one of the most important end points assessed in this study was the amount of reversible perfusion defect at follow-up. The end point of contractility is more difficult to evaluate in light of the differences between the groups at baseline; however, changes in opposite directions occurred at follow-up. In addition, the slightly better LVEFs and smaller hearts should logically have biased results against the cell-treated group.

Although the mechanisms by which cell therapy confers clinical benefit are not well understood, correlation between cell phenotype subpopulation analysis and long-term clinical outcomes is beyond the scope of the present study. Future analyses will be performed in this regard when longer-term follow-up is available.

The treatment of patients with heart failure has become increasingly important given the growing number of cases and their economic impact on the healthcare system.[25,26] More aggressive and widespread therapy in patients with chronic, ischemic heart failure will ultimately lead to a population harboring more advanced disease with a potential yearly mortality rate as high as 50%.[27] For these patients, therapeutic options remain limited. The very high-risk nature of the patient population represented in our study cohort is underscored by the fact that there was a death in both the control and the treatment groups. However, the significant improvement in LVEF noted in the treatment group on angiographic follow-up at 4 months (from 20% to 29%) may imply an improved clinical state and, it is hoped, provide some reduction in risks for the future.[28]

Conclusion

In this initial prospective, nonrandomized, open-label study in no-other-option coronary artery disease patients with LV dysfunction, we noted improvement in symptoms, cardiac function, and perfusion with transendocardial ABMMNC therapy, without any clinical evidence of significant harm from the procedure itself. We believe there may be clinical potential for this relatively novel therapy. Further investigation in a larger, randomized trial is warranted.

Acknowledgments

A NOGA mapping system and catheters were provided by Cordis Corporation (Miami Lakes, Fla).

We thank Rita Weiler, Ana Cristina Reis, RN, and Patricia Souza, RN, for their enthusiastic support and coordination of the study patients; Cristine Rutherford for the psychological assessment and support of the patients; David R. Buskohl, Mark Martin, and Jacqueline Grant for their outstanding technical support; and Marianne Mallia, ELS, for editorial assistance in the preparation of the manuscript.

References

1. Orlic D, Kajstura J, Chimenti S, et al. Bone marrow cells regenerate infarcted myocardium. *Nature*. 2001;410:701–705.
2. Kocher AA, Schuster MD, Szabolcs MJ, et al. Neovascularization of ischemic myocardium by human bone-marrow–derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function. *Nat Med*. 2001;7:430–436.
3. Kawamoto A, Gwon HC, Iwaguro H, et al. Therapeutic potential of ex vivo expanded endothelial progenitor cells for myocardial ischemia. *Circulation*. 2001;103:634–637.
4. Fuchs S, Baffour R, Zhou YF, et al. Transendocardial delivery of autologous bone marrow enhances collateral perfusion and regional function in pigs with chronic experimental myocardial ischemia. *J Am Coll Cardiol*. 2001;37:1726–1732.
5. Strauer BE, Brehm M, Zeus T, et al. Repair of infarcted myocardium by autologous intracoronary mononuclear bone marrow cell transplantation in humans. *Circulation*. 2002;106:1913–1918.

6. Assmus B, Schächinger V, Teupe C, et al. Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction (TOPCARE-AMI). *Circulation*. 2002;106:3009–3017.
7. Tse HF, Kwong YL, Chan JKF, et al. Angiogenesis in ischaemic myocardium by intramyocardial autologous bone marrow mononuclear cell implantation. *Lancet*. 2003;361:47–49.
8. Gibbons RJ, Balady GJ, Bricker TJ, et al. ACC/AHA 2002 guideline update for exercise testing: summary article. A report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Committee to Update the 1997 Exercise Testing Guidelines). *J Am Coll Cardiol*. 2002;40:1531–1540.
9. Coutinho LH, Gilleece MH, de Wynter EA, et al. Clonal and long-term cultures using human bone marrow. In: Testa NG, Molineux G, eds. *Haemopoiesis: A Practical Approach*. New York, NY: Oxford University Press, 1993:84–85.
10. Castro-Malaspina H, Gay RE, Resnick G, et al. Characterization of human bone marrow fibroblast colony-forming cells (CFU-F) and their progeny. *Blood*. 1980;56:289–301.
11. Perin EC, Silva GV, Sarmento-Leite R, et al. Assessing myocardial viability and infarct transmurality with left ventricular electromechanical mapping in patients with stable coronary artery disease: validation by delayed-enhancement magnetic resonance imaging. *Circulation*. 2002; 106:957–961.
12. Perin EC, Silva GV, Leite RS. Left ventricular electromechanical mapping as a diagnostic method. In: Abela GS, ed. *Myocardial Revascularization: Novel Percutaneous Approaches*. New York, NY: Wiley-Liss; 2001:183–195.
13. Kaminsky LA, Whaley MH. Evaluation of a new standardized ramp protocol: the BSU/Bruce Ramp protocol. *J Cardiopulm Rehabil*. 1998; 18:438–444.
14. American College of Sport Medicine. *Guidelines for Exercise Testing and Exercise Prescription*. 6th ed. Philadelphia, Pa: Lippincott Williams & Wilkins; 2000.
15. Garcia EV, Cooke CD, Van Train KF, et al. Technical aspects of myocardial SPECT imaging with technetium-99m sestamibi. *Am J Cardiol*. 1990;66:23E–31E.
16. Van Train K, Areeda J, Garcia EV, et al. Quantitative same-day rest stress technetium-99 m sestamibi SPECT: definition and validation of stress normal limits and criteria for abnormality. *J Nucl Med*. 1993;34: 1494–1502.
17. Van Train K, Garcia EV, Maddahi J, et al. Multicenter trial validation for quantitative analysis of same-day rest-stress technetium-99m sestamibi myocardial tomograms. *J Nucl Med*. 1994;35:609–618.
18. Dodge HT, Sandler H, Ballew DW, et al. The use of biplane angiography for the measurement of left ventricular volume in man. *Eur Heart J*. 1960;60:762–776.
19. Toma C, Pittenger MF, Cahill KS, et al. Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. *Circulation*. 2002;105:93–98.
20. Asahara T, Murohara T, Sullivan A, et al. Isolation of putative progenitor endothelial cells for angiogenesis. *Science*. 1997;275:964–967.
21. Badorff C, Brandes RP, Popp R, et al. Transdifferentiation of blood-derived human adult endothelial progenitor cells into functionally active cardiomyocytes. *Circulation*. 2003;107:1024–1032.
22. Bikfalvi A, Han ZC. Angiogenic factors are hematopoietic factors and vice versa. *Leukemia*. 1994;8:523–529.
23. Shintani S, Murohara T, Ikeda H, et al. Mobilization of endothelial progenitor cells in patients with acute myocardial infarction. *Circulation*. 2001;103:2776–2779.
24. Wolf T, Gepstein L, Dror V, et al. Detailed electromechanical mapping accurately predicts the transmural extent of myocardial infarction. *J Am Coll Cardiol*. 2001;37:1590–1597.
25. American Heart Association. *2000 Heart and Stroke Statistical Update*. Dallas, Tex: American Heart Association; 2001.
26. O'Connell JB, Birstow MR. Economic impact of heart failure in the United States: time for a different approach. *J Heart Lung Transplant*. 1994;13:S107–S112.
27. Califf RM, Adams KF, McKenna WJ, et al. A randomized controlled trial of epoprostenol therapy for severe congestive heart failure: the Flolan International Randomized Trial (FIRST). *Am Heart J*. 1997;134:44–54.
28. Marantz PR, Tobin JN, Wassertheil-Smoller S, et al. Prognosis in ischemic heart disease. Can you tell as much at the bedside as in the nuclear laboratory? *Arch Intern Med*. 1992;152:2433–2437.

Circulation
JOURNAL OF THE AMERICAN HEART ASSOCIATION

Transendocardial Autologous Bone Marrow Mononuclear Cell Injection in Ischemic Heart Failure: Postmortem Anatomicopathologic and Immunohistochemical Findings

Hans F.R. Dohmann, Emerson C. Perin, Christina M. Takiya, Guilherme V. Silva, Suzana A. Silva, Andre L.S. Sousa, Claudio T. Mesquita, Maria-Isabel D. Rossi, Bernardo M.O. Pascarelli, Isabella M. Assis, Helio S. Dutra, João A.R. Assad, Rodrigo V. Castello-Branco, Cantidio Drummond, Hans J.F. Dohmann, James T. Willerson and Radovan Borojevic

Circulation 2005;112;521-526; originally published online Jul 18, 2005;
DOI: 10.1161/CIRCULATIONAHA.104.499178
Circulation is published by the American Heart Association. 7272 Greenville Avenue, Dallas, TX 72514
 Print ISSN: 0009-7322. Online ISSN: 1524-4539

Transendocardial Autologous Bone Marrow Mononuclear Cell Injection in Ischemic Heart Failure

Postmortem Anatomicopathologic and Immunohistochemical Findings

Hans F.R. Dohmann, MD*; Emerson C. Perin, MD, PhD*; Christina M. Takiya, MD, PhD; Guilherme V. Silva, MD; Suzana A. Silva, MD; Andre L.S. Sousa, MD; Claudio T. Mesquita, MD, PhD; Maria-Isabel D. Rossi, PhD; Bernardo M.O. Pascarelli, MD; Isabella M. Assis, MD; Helio S. Dutra, PhD; João A.R. Assad, MD; Rodrigo V. Castello-Branco, MD; Cantidio Drummond, MD; Hans J.F. Dohmann, MD, PhD; James T. Willerson, MD; Radovan Borojevic, PhD

Background—Cell-based therapies for treatment of ischemic heart disease are currently under investigation. We previously reported the results of a phase I trial of transendocardial injection of autologous bone marrow mononuclear (ABMM) cells in patients with end-stage ischemic heart disease. The current report focuses on postmortem cardiac findings from one of the treated patients, who died 11 months after cell therapy.

Methods and Results—Anatomicopathologic, morphometric, and immunocytochemical findings from the anterolateral ventricular wall (with cell therapy) were compared with findings from the interventricular septum (normal perfusion and no cell therapy) and from the inferoposterior ventricular wall (extensive scar tissue and no cell therapy). No signs of adverse events were found in the cell-injected areas. Capillary density was significantly higher ($P<0.001$) in the anterolateral wall than in the previously infarcted tissue in the posterior wall. The prominent vasculature of the anterolateral wall was associated with hyperplasia of pericytes, mural cells, and adventitia. Some of these cells had acquired cytoskeletal elements and contractile proteins (troponin, sarcomeric α-actinin, actinin), as well as the morphology of cardiomyocytes, and appeared to have migrated toward adjacent bundles of cardiomyocytes.

Conclusions—Eleven months after treatment, morphological and immunocytochemical analysis of the sites of ABMM cell injection showed no abnormal cell growth or tissue lesions and suggested that an active process of angiogenesis was present in both the fibrotic cicatricial tissue and the adjacent cardiac muscle. Some of the pericytes had acquired the morphology of cardiomyocytes, suggesting long-term sequential regeneration of the cardiac vascular tree and muscle. (***Circulation.*** **2005;112:521-526.)**

Key Words: angiogenesis ■ stem cells ■ heart failure ■ revascularization ■ ischemia

The role of cell-based therapy for the treatment of ischemic heart disease is currently under investigation. In view of the myocardium's limited capacity to regenerate spontaneously after an ischemic injury, the therapeutic use of exogenous progenitor cells has recently gained increasing interest. In vitro demonstration of functional cardiomyocyte differentiation from bone marrow–derived progenitor cells[1,2] has prompted in vivo studies in animal models, and promising results have been obtained in the repair and regeneration of acute and chronic cardiac muscle lesions. Several types of progenitor cells have been used in experimental models, including bone marrow–derived endothelial and blood cell progenitors, as well as bone marrow mesenchymal progenitors.[3–6]

In humans, similar attempts have been made with surgical, intracoronary, or transendocardial introduction of bone marrow–derived cells to improve cardiac lesions.[7,8] Our group recently reported the results of the first phase I human trial of transendocardial injection of autologous bone marrow mononuclear (ABMM) cells in patients with end-stage ischemic heart disease.[9] We observed a significant increase in perfusion, contractility of ischemic myocardial segments, and

Received March 31, 2004; de novo received August 13, 2004; revision received January 28, 2005; accepted February 23, 2005.

From the Hospital Procardiaco (H.F.R.D., S.A.S., A.L.S.S., C.T.M., J.A.R.A., R.V.C.B., C.D., H.J.F.D.), Rio de Janeiro, Brazil; the Texas Heart Institute at St. Luke's Episcopal Hospital (E.C.P., G.V.S., J.T.W.), Houston, Tex; and the Institute of Biomedical Sciences and Clementino Fraga Filho Hospital, Federal University (C.M.T., M.-I.D.R., B.M.O.P., I.M.A., H.S.D., R.B.), Rio de Janeiro, Brazil.

*Drs Dohmann and Perin are coprincipal investigators.

Correspondence to Hans F.R. Dohmann, MD, Rua General Polidoro 192, 22080-000 Rio de Janeiro, Brazil (e-mail diretoria.cientifica@procardiaco.com.br), or Emerson C. Perin, MD, 6624 Fannin, Suite 2220, Houston TX 77030 (e-mail eperin@crescentb.net).

Circulation is available at http://www.circulationaha.org DOI: 10.1161/CIRCULATIONAHA.104.499178

Phenotype and Functional Characterization of 3×10^7 Cells Injected via a Transendocardial Route*

Phenotype, % in ABMM cell fraction	
$CD45^{Lo}$ $CD34^+$	3.2
$CD45^{Lo}$ $CD34^+$ $HLA\text{-}DR^-$	0.2
T cells ($CD4^+$)	29.3
T cells ($CD8^+$)	24.4
B cells ($CD19^+$)	8.7
NK cells ($CD56^+$)	0.7
Monocytes ($CD14^{hi}$)	13.7
Functional assay, cell No./10^6 ABMM cells	
CFU-GM	802
CFU-F	1

NK indicates natural killer; CFU-F, colony-forming-unit fibroblasts; and CFU-GM, colony-forming-unit granulocyte/macrophages.

*After mononuclear fraction purification, cell viability was 98.1%.

functional capacity of the cell-injection recipients. This report presents postmortem cardiac findings from one of these patients.

Case Report

The patient was a 55-year-old man with ischemic cardiomyopathy and 2 previous myocardial infarctions (in 1985 and 2000). He began to have symptoms of congestive heart failure 2 years before study enrollment. One year before enrollment, the patient had an ischemic stroke with mild residual right hemiparesis and resultant episodes of chronic tonic-clonic seizures. His risk factors for coronary artery disease included diabetes mellitus type II, hypertension, and hypercholesterolemia.

The patient's functional capacity was evaluated at baseline by means of a ramp treadmill protocol[10] with a peak maximal oxygen consumption ($\dot{V}o_2max$) of 15.9 $mL \cdot kg^{-1} \cdot min^{-1}$ and a workload of 4.51 metabolic equivalents (METs). A baseline single-photon-emission computed tomography (SPECT) perfusion study showed a partially reversible perfusion defect in the anterolateral wall, a fixed perfusion defect (scar) in the inferior and posterior walls, and normal perfusion in the septal wall.

Cardiac catheterization revealed a left ventricular ejection fraction of 11%, a 70% ostial and an 85% middle stenosis of the left anterior descending (LAD) coronary artery, an 80% proximal lesion of the left circumflex (LCx) coronary artery, and total occlusion of the first obtuse marginal artery and right coronary artery. The distal segments of the LAD and LCx were diffusely diseased. Owing to the severity and extent of the patient's coronary disease, he was not considered a candidate for surgical or interventional procedures. At enrollment in our study, he was in New York Heart Association (NYHA) functional class III and Canadian Cardiovascular Society (CCS) angina class III. His serum C-reactive protein level, complete blood count, creatine kinase level, and troponin level were normal at baseline.

The patient received a total of 3×10^7 ABMM cells (the Table) that had been harvested 2 hours before the procedure. With the guidance of electromechanical mapping,[11,12] the cells were injected transendocardially into the anterolateral wall of the left ventricle. No periprocedural complications were observed.

Noninvasive follow-up evaluation was performed 2 and 6 months after cell therapy. Invasive follow-up evaluation, with cardiac catheterization, was performed at 4 months and revealed no change in coronary anatomy. Symptomatic and functional improvements were noted because the patient returned to NYHA and CCS class I. Holter monitoring showed no malignant ventricular arrhythmias, and signal-averaged ECG parameters remained stable. There was no change in the patient's medications after cell therapy. There was no change in the global ejection fraction or left ventricular volume on echocardiography. The wall-motion index score (on 2-dimensional echocardiography) improved from 1.94 to 1.65 as contractility increased in 5 segments adjacent to the injected area. Myocardial perfusion, as assessed by SPECT, improved in the anterolateral wall. Mechanical data derived from SPECT showed improvements in regional ejection fraction, wall motion, and thickening. In addition, during ramp treadmill testing, the $\dot{V}o_2max$ increased from 15.8 to 25.2 $mL \cdot kg^{-1} \cdot min^{-1}$, and the METs increased from 4.51 to 7.21 at 2 months. At 6-month follow-up testing, the $\dot{V}o_2max$ reached 31.6 $mL \cdot kg^{-1} \cdot min^{-1}$, and the METs was 9.03.

From 6 to 11 months after the cell injection procedure, the patient's cardiovascular condition remained stable. At 11 months, however, he had a tonic-clonic seizure at home and was found in cardiopulmonary arrest by family members.

Methods

After signed, informed consent was given by the family, an autopsy was performed, including morphological and immunocytochemical analysis of the heart. This report presents the anatomicopathologic findings about the infarcted areas of the anterolateral ventricular wall, which were the areas that had received bone marrow cell injections. The histological findings from this region were compared with findings from within the interventricular septum (which had normal perfusion in the central region and no cell therapy) and findings from the previously infarcted inferoposterior ventricular wall (which had extensive scarring and no cell therapy).

Immunocytochemical analysis of paraffin sections was performed with antibodies against factor VIII–related antigen (A0082, Dako), vimentin (M0725, Dako), smooth muscle α-actin (M0851, Dako), and CD34 and Ki-67 (NCL-L-End and NCL-Ki67MM1 respectively, Novocastra). Antibodies were reacted with Dako's EnVision+ System/HRP, with diaminobenzidine as a chromogen. Frozen sections were fixed, permeated with acetone, and incubated with antibodies for troponin T (T6277, Sigma), smooth muscle α-actin, sarcomeric actinin (A7811, Sigma), and desmin (D1033, Sigma). Antibodies were revealed with anti-mouse or anti-rabbit IgG, $F(ab)_2$ fragment, conjugated to fluorescein isothiocyanate (1814192 and 1238833, respectively, Boehringer-Mannheim), and counterstained with a 0.1‰ solution of Evans blue dye (Merck).

Capillary density was monitored by using computerized image analysis (Image-Pro Plus, MediaCybernetics) of randomly selected fields in sections stained with hematoxylin and reacted with antibody for factor VIII–related antigen ($n=108$) and randomly selected fields in sections reacted with antibodies for smooth muscle α-actin ($n=96$). Transverse sections of capillaries identified by staining for factor VIII and pericyte-containing capillaries identified by staining for smooth muscle α-actin were quantified separately. Results were expressed as the mean number of capillaries per square millimeter in the case of factor VIII–stained slides or the number of capillaries containing pericytes in α-smooth-muscle-actin–stained slides. Larger vessels identified by a continuous wall of smooth muscle actin–positive mural cells were excluded. Differences between the anterolateral, septal, and posterior walls were assessed with Kruskal-Wallis ANOVA and the

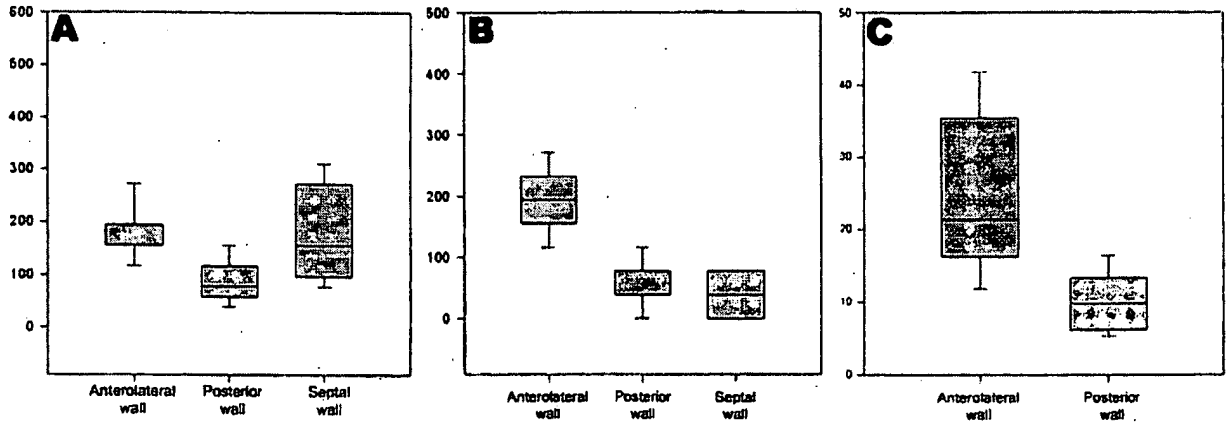


Figure 1. Number of capillaries per mm² in anterolateral, posterior, and septal walls of studied heart. A, Anti-factor VIII–associated antigen counterstained with hematoxylin. B, Anti-smooth muscle α-actin antigen counterstained with hematoxylin. C, Capillaries reacted with anti-factor VIII–associated antigen inside fibrotic areas only in anterolateral and posterior walls. (n=108 microscope fields for A; 96 microscope fields for B; and 40 microscopic fields for C.) Differences were statistically significant among all groups in pairwise comparisons ($P<0.05$, Newman-Keuls method) for A and B. Differences were significantly different ($P<0.05$) between anterolateral and posterior walls in Mann-Whitney rank-sum test for C.

Student-Newman-Keuls method for pairwise multiple comparison. Results were considered significant if P was <0.05.

Evaluation of the capillary density inside the fibrotic areas within the cell-treated anterolateral wall versus the nontreated posterior wall was performed in 40 selected fields inside the fibrotic scars, excluding the regions containing cardiomyocytes. Microscope fields (at ×100) of factor VIII–stained slides were digitized, and the number of transverse sections of capillaries per square millimeter of fibrotic zones was assessed. Differences between the treated infarcted zones and the nontreated fibrotic wall were assessed by the Mann-Whitney rank-sum test. Results were considered significant if P was <0.05.

Results

Anatomopathologic Findings

The heart weighed 765 g. There was severe arteriosclerosis with subocclusive calcified atheromata in all coronary arteries, calcification of the pulmonary artery, and moderate atheromatosis of the aorta. The heart cavities were dilated, with hypertrophic walls. There was no evidence of any acute injury or of lesions that could be related to cell injections. A generalized, homogeneous endocardial opacification, affecting all the cardiac internal surfaces, was identified on histological examination as diffuse fibroelastic hyperplasia of the endocardium. Minute focal and punctate scars were observed, mainly in the posterior and anterolateral walls.

The apical zone was thinned and fibrotic. The posterior and apical regions had dense, fibrotic, well-circumscribed scars that separated cardiomyocyte bundles. The septal wall exhibited focal scars interspersed with cardiac fibers in the regions adjacent to the anterior and posterior ventricular walls, but it was devoid of fibrosis in the central region.

The anterolateral ventricular wall that received cell injections had elongated, irregular, and parallel reddish areas throughout. In the same wall, in adjacent regions that did not receive injections, the density and morphology of the fibrotic scars were similar to those of the posterior wall, suggesting that no overt differences were present among the different infarcted areas before cell injections.

Morphometric Analysis

The capillary density was significantly higher in the areas of the anterolateral ventricular walls that received cell injections than in the previously infarcted posterior wall ($P<0.0001$) (Figure 1A). The median capillary density in the anterolateral wall was apparently similar to that in the septal wall. However, the broad dispersion of the septal wall data, which may have been due to fibrotic areas in regions close or adjacent to the ventricular walls, generated a statistically significant difference between these 2 groups.

The density of capillaries that contained smooth muscle α-actin–positive cells within their walls was also assessed (Figure 1B). The number of such vessels was higher in the anterolateral wall than in the septal and posterior walls ($P<0.0001$). Larger vessels identified by a continuous wall of smooth muscle α-actin–positive mural cells were not included in these analyses. The capillary density was significantly higher within fibrotic areas of the anterolateral wall than within fibrotic areas of the posterior wall ($P<0.0001$) (Figure 1C).

Histological Findings

The anterolateral wall showed irregular, pale regions of fibrotic tissue intercalated with dark regions of cardiac muscle arranged in roughly parallel, interspersed bands, perpendicular to the ventricular wall plane (Figure 2A). No abnormal cell organization, growth, or differentiation or signs of previous focal necrosis, inflammatory reactions, or tissue repair were found in the region that had received cell injections. Inside the fibrotic tissue, trichrome and picrosirius collagen staining disclosed regions with decreased collagen density, in which a rich vascular tree was present. The anterolateral wall also showed larger central vessels that ramified into smaller ones, parallel to the cardiomyocyte bundles (Figure 2B). In the anterolateral wall, the peripheral zone of fibrotic areas merged into the cardiomyocyte layer and lacked well-defined limits, unlike the fibrotic areas observed in the posterior wall (Figure 2C). No fibrotic tissue was seen in the central area of the septal wall (Figure 2D).

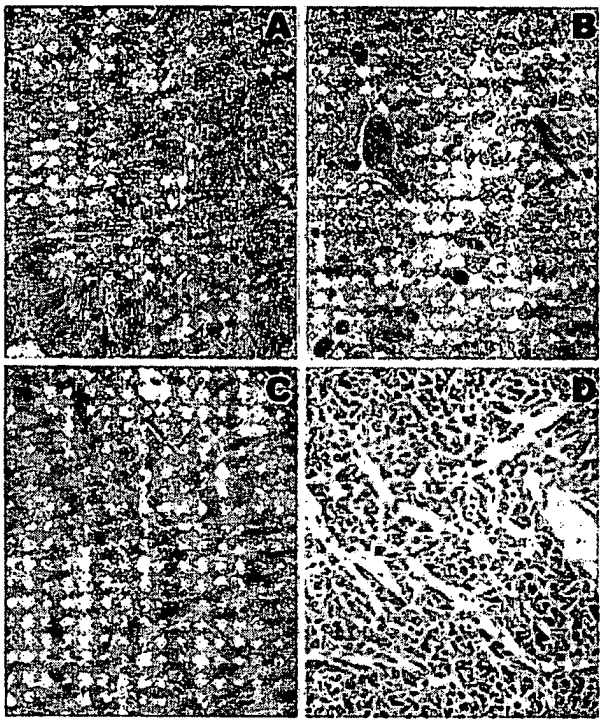


Figure 2. Gomori trichrome stain of anterolateral (A, B), posterior (C), and septal (D) walls. Increased vascular tree is present in B. Original magnification is ×40 in A, B, and D; ×100 in C.

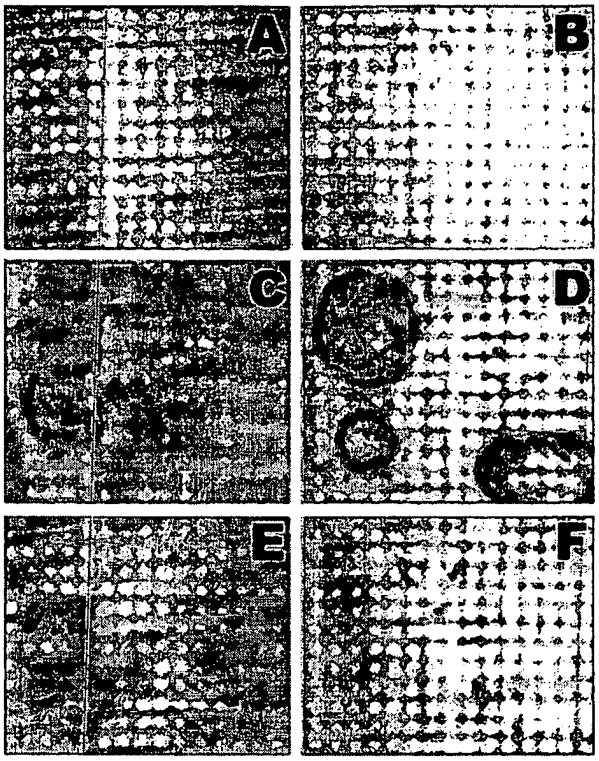


Figure 3. Immunocytochemical identification of factor VIII–associated antigen (A, B) and smooth muscle α-actin (C–E) in blood vessel walls of septal (A) and anterolateral (B–E) regions of studied heart, depicting increased vascular density (B) and hyperplasia of perivascular and mural cells (C–E). Ki67 reactivity was rarely present in perivascular cells of anterolateral wall (F). Original magnification ×40 in A and B, ×400 in C and D, and ×1000 in E and F.

Inflammatory cells were rare in the perivascular region: There were occasional isolated small groups of lymphocytes and, very rarely, granulocytes. At the interface between fibrotic tissue and cardiomyocyte bundles, 2 gradients merged: the decreasing blood vessel diameter and the increasing cardiomyocyte size. Very small cardiomyocytes were seen isolated in the fibrotic matrix adjacent to capillaries in the anterolateral wall, together with a progressively increasing number of fibroblastoid cells that were isolated or interspersed in small groups among the cardiomyocyte bundles.

Immunocytochemistry Findings

Immunocytochemical labeling of factor VIII–associated antigen identified a thin endothelial layer of blood vessels in the posterior, septal (Figure 3A), and anterolateral (Figure 3B) ventricular walls. In the anterolateral wall, neither factor VIII nor CD34 was found in the fibroblastoid cell population inside the fibrotic matrix. In the posterior ventricular wall and septum, smooth muscle α-actin was readily identified in blood vessel wall cells. This protein was present both in pericytes and in the smooth muscle cells of the thin vessel wall layer (Figure 3C) in the anterolateral wall. The vascular tree of the anterolateral wall showed intense labeling in the blood vessel walls, which had a marked hypertrophy of smooth muscle cells (Figure 3D). The same staining pattern was present in isolated cells located in the perivascular position and in the adjacent region among cardiomyocytes and fibrotic matrix (Figure 3E). Vimentin was present in the endothelial layer of the anterolateral wall, in the perivascular cells, and in cells adjacent to or in close contact with the cardiomyocytes (Figure 4A). These cells frequently formed an extensive network that permeated the fibrotic matrix and the interstitial space among cardiomyocytes (Figure 4B).

Desmin was identified in the same cell population. Desmin labeling was less intense in the vascular wall cells and isolated perivascular cells and was more intense in the cells adjacent to cardiomyocytes. On sections perpendicular to the main cardiomyocyte axis, thin desmin-positive fibrils were observed mainly in the submembrane region; on longitudinal sections, a typical transverse banded pattern of desmin was observed (Figure 4C and 4D). Among cardiomyocytes, some of the small cells had strong, peripheral desmin-stained areas (Figure 4D).

In vascular and perivascular cells in the posterior wall and septum, troponin labeling was negative. In the anterolateral wall, troponin labeling was negative in capillary walls but was positive in the adjacent pericapillary pericytes and in cells migrating into the pericapillary matrix (Figure 4E). In larger vessels, troponin-positive cells were observed in the outer cell layers and adventitia, occasionally forming a continuous troponin-positive cell layer around the vessel (Figure 4F). Isolated cells or small groups of troponin-positive cells were found in the area between the fibrotic tissue and cardiomyocytes and inside the adjacent cardiomyocyte bundles. The intensity of labeling increased in the proximity of cardiomyocytes, where some small fibroblastoid cells disclosed a bright cytoplasm

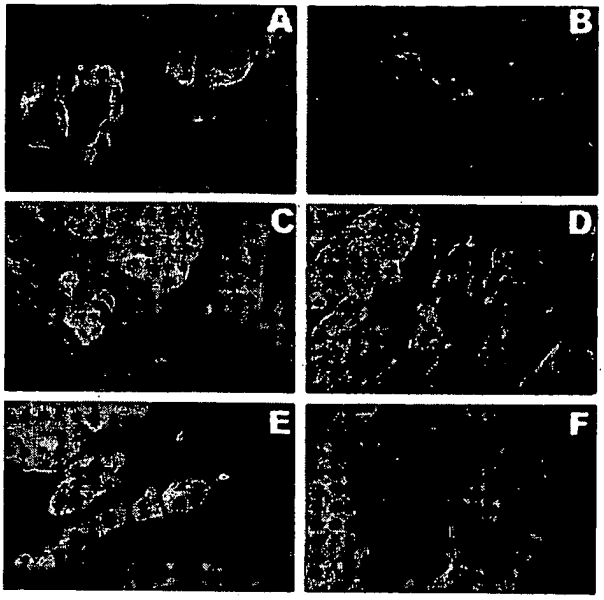


Figure 4. Anterolateral wall that received cell injection therapy. A and B, Immunostaining for vimentin depicted positive reaction in vascular wall and in fibroblastoid interstitial cells. C and D, Immunostaining for desmin showed small groups of intensely reactive cells between blood vessels and cardiomyocytes (C) and small cells inside cardiomyocyte bundles with typical striated cytoskeleton (D). E and F, Immunostaining for troponin showed positive reaction in all mural cells of medium-sized blood vessel. Original magnification is ×1000 in A and F; ×400 in B–E.

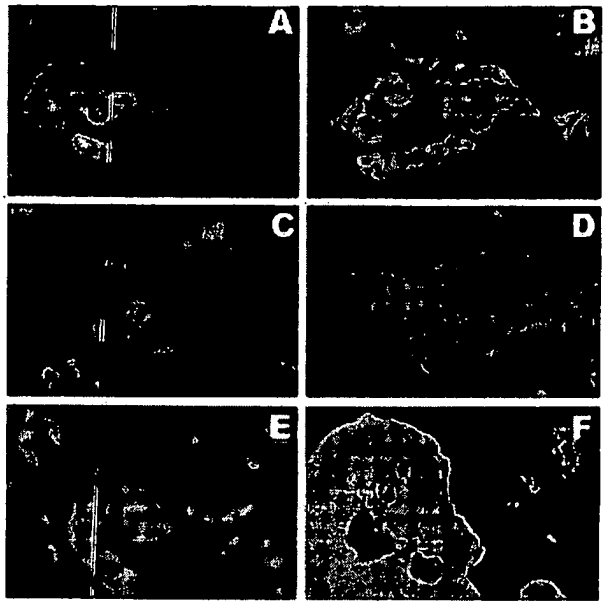


Figure 5. Anterolateral wall that received cell injection therapy. A, Immunostaining for troponin depicted small cardiomyocyte-like cells with intense reaction in peripheral cell area. B–F, Immunostaining for sarcomeric actinin depicted reactivity in mural cells of blood vessel (B–E) and isolated cells among cardiomyocytes with actinin organization similar to that of sarcomeres (E, F). Original magnification is ×400 in A and F; ×1000 in B–E.

homogeneously labeled for troponin (Figure 5A). Occasionally, such cells had an increased volume, with troponin labeling restricted to the periphery; the central area was filled by a troponin-negative cytoskeleton similar to the desmin-stained areas in small cardiomyocytes. In mature cardiomyocytes, troponin-specific antibody labeled the peripheral filamentous and sarcomeric cytoskeleton.

Labeling of sarcomeric actinin was similar to that of troponin. However, both pericapillary pericytes and mural blood vessel cells in the anterolateral region were negative for sarcomeric actinin in blood vessels that were deeply embedded in the fibrotic scar matrix and that remained distant from cardiomyocyte bundles. The same cells located in vessels adjacent to or embedded between the cardiomyocyte bundles were positive for sarcomeric actinin, as were the isolated cells or small groups of fibroblastoid cells (Figures 5B and 5C). Some of these cells had increased in size and, in their central region, disclosed sarcomeric actinin that was already organized in the typical banded pattern of sarcomeres (Figures 5D and 5E). In this central region, isolated cells barely larger than pericytes could be observed; only a few sarcomeres were present, suggesting that those isolated cells had acquired some cardiomyocyte characteristics (Figure 5F).

The Ki67 antibody, which identifies cells actively engaged in replication, reacted only rarely with endothelial cells in the posterior wall. In the anterolateral region, the Ki67 antibody also reacted with pericapillary pericytes and with isolated fibroblastoid cells in the surrounding fibrotic matrix (Figure 3F). The overall cell reactivity with Ki67 antibody was relatively low.

Discussion

Accumulating evidence from both experimental animal studies[4–6] and human trials[7–9] indicates that ABMM cell therapy improves myocardial perfusion in patients with ischemic heart disease. At the same time, clinical stem cell therapy research is focusing more on safety than on efficacy. The present report describes the postmortem study of one patient who underwent transendocardial injection of ABMM cells. Accordingly, the major findings in this report pertain to the procedure's safety: No abnormal or disorganized tissue growth, no abnormal vascular growth, and no enhanced inflammatory reactions were observed. In addition, some intriguing histological and immunohistochemical findings were documented: (1) There was a higher capillary density in the cell-treated area than in nontreated areas of the heart. (2) A proliferation of smooth muscle α-actin–positive pericytes and mural cells was noted. (3) The aforementioned cells expressed specific cardiomyocyte proteins.

In the postnatal period, new blood vessels form through either vasculogenesis or angiogenesis, in which proliferation of endothelial cells is followed by remodeling of the extracellular matrix and proliferation of blood wall cells.[13–15] Endothelial cells can result from bone marrow–derived progenitors (postnatal vasculogenesis) or from the migration and proliferation of endothelial cells from existing vessels (angiogenesis).[16] Mural cells such as pericytes and smooth muscle cells can be derived from bone marrow mesenchymal cells (stroma), myofibroblasts, and/or fibroblasts.[17] In the neoangiogenic process, pericytes are derived either from cells of adjacent tissues (mobilized by growth factors produced by endothelial cells) or from proliferation of adventitial and pericapillary pericytes and

their distal gliding on the abluminal side of the growing blood vessel's basement membranes.[13] The alternative origination of pericytes from mesenchymal stem cells has been proposed and preliminarily confirmed in experimental models.[18] Pericytes may be essential to achieve a physiological angiogenic process with resultant durable blood vessels. In the present case, when compared with the noninjected regions, the cell-injected wall had marked hyperplasia of pericytes and mural cells. The observed hypertrophic pericytes displayed 2 characteristics: First, although still located in the vascular wall, they expressed specific myocardial proteins and second, they were found in locations that suggested detachment, having migrated into the adjacent tissue and reached proximal cardiomyocytes that were either isolated or in small cell clumps. Closer to cardiomyocytes, the expression of myocardial proteins was enhanced, yielding brighter immunostaining throughout the whole cytoplasm. The significance of these findings remains to be established. However, within the posterior wall, none of the findings was seen, and small blood vessels could only rarely be found.

Notwithstanding the aforedescribed data, the present report has limitations that severely restrict our ability to make conclusions about the role of ABMM cells in myocardial regeneration. The findings could have occurred by chance. It is impossible to exclude the influence of a natural recovery process as the cause for the difference in vascular density between cell-treated and nontreated areas. Comparisons of capillary density among different sections of wall were based on specimens from a single patient. Moreover, this is an isolated, uncontrolled case involving late events after injection of unlabeled cells; it precluded the use of any imaging technique that could have helped to colocalize and identify the presence of stem cell direct descendants within the vessel wall or myocardium. Therefore, the significant difference in vascular density between cell-treated and nontreated areas cannot be extrapolated to a larger population of similar patients. However, the increased vascular density within the cell-injected anterolateral wall accompanied that wall's improvement in perfusion as assessed by SPECT, whereas all other walls remained unchanged.

Conclusion

At 11-month follow-up evaluation, stem cell therapy was not associated with any adverse histological findings. Morphological and immunohistochemical analysis of the area that underwent ABMM cell implantation suggested that that area had more capillaries than nontreated areas and that ABMM cell therapy was associated with hyperplasia of pericytes, mural cells, and adventitia. Some of these cells had acquired cytoskeletal elements and contractile proteins (desmin, troponin, and sarcomeric actinin).

Acknowledgments

The authors thank L. Maximilian Buja, MD, and Silvio Litovsky, MD, for reviewing the pathological findings; Marianne Mallia-Hughes, ELS, and Virginia Fairchild of the Section of Scientific Publications of the Texas Heart Institute, for editorial assistance; and William K. Vaughn, PhD, for assistance with statistical questions.

References

1. Hakuno D, Fukuda K, Makino S, Konishi F, Tomita Y, Manabe T, Suzuki Y, Umezawa A, Ogawa S. Bone marrow-derived regenerated cardiomyocytes (CMG cells) express functional adrenergic and muscarinic receptors. *Circulation*. 2002;105:380–386.
2. Makino S, Fukuda K, Miyoshi S, Konishi F, Kodama H, Pan J, Sano M, Takahashi T, Hori S, Abe H, Hata J, Umezawa A, Ogawa S. Cardiomyocytes can be generated from marrow stromal cells in vitro. *J Clin Invest*. 1999;103:697–705.
3. Orlic D, Kajstura J, Chimenti S, Jakoniuk I, Anderson SM, Li B, Pickel J, McKay R, Nadal-Ginard B, Bodine DM, Leri A, Anversa P. Bone marrow cells regenerate infarcted myocardium. *Nature*. 2001;410:701–705.
4. Jackson KA, Majka SM, Wang H, Pocius J, Hartley CJ, Majesky MW, Entman ML, Michael LH, Hirschi KK, Goodell MA. Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells. *J Clin Invest*. 2001;107:1395–1402.
5. Nishida M, Li TS, Hirata K, Yano M, Matsuzaki M, Humano K. Improvement of cardiac function by bone marrow cell implantation in a rat hypoperfusion heart model. *Ann Thorac Surg*. 2003;75:768–773.
6. Olivares EL, Ribeiro VP, João PS, Ribeiro KC, Mattos EC, Goldenberg RC, Mill JG, Dohmann HF, dos Santos RR, de Carvalho AC, Masuda MO. Bone marrow stromal cells improve cardiac performance in healed infarcted rat hearts. *Am J Physiol Heart Circ Physiol*. 2004;287:H464–H470.
7. Assmus B, Schächinger V, Teupe C, Britten M, Lehmann R, Dobert N, Grunwald F, Aicher A, Urbich C, Martin H, Hoelzer D, Dimmeler S, Zeiher AM. Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction (TOPCARE-AMI). *Circulation*. 2002;106:3009–3017.
8. Strauer BE, Brehm M, Zeus T, Kostering M, Hernandez A, Sorg RV, Kogler G, Wernet P. Repair of infarcted myocardium by autologous intracoronary mononuclear bone marrow cell transplantation in humans. *Circulation*. 2002;106:1913–1918.
9. Perin EC, Dohmann HFR, Borojevic R, Silva SA, Sousa AL, Mesquita CT, Rossi MI, Carvalho AC, Dutra HS, Dohmann HJ, Silva GV, Belem L, Vivacqua R, Rangel FO, Esporcatte R, Geng YJ, Vaughn WK, Assad JA, Mesquita ET, Willerson JT. Transendocardial, autologous bone marrow cell transplantation for severe, chronic ischemic heart failure. *Circulation*. 2003;107:2294–2302.
10. Gibbons R, Balady GJ, Bricker JT, Chaitman BR, Fletcher GF, Froelicher VF, Mark DB, McCallister BD, Mooss AN, O'Reilly MG, Winters WL, Gibbons RJ, Antman EM, Alpert JS, Faxon DP, Fuster V, Gregoratos G, Hiratzka LF, Jacobs AK, Russell RO, Smith SC; American College of Cardiology/American Heart Association Task Force on Practice Guidelines. Committee to Update the 1997 Exercise Testing Guidelines. ACC/AHA 2002 guideline update for exercise testing: summary article. A report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Committee to Update the 1997 Exercise Testing Guidelines). *J Am Coll Cardiol*. 2002;40:1531–1540.
11. Perin EC, Silva G, Sarmento-Leite R, Sousa AL, Howell M, Muthupillai R, Lambert B, Vaughn WK, Flamm SD. Assessing myocardial viability and infarct transmurality with left ventricular electromechanical mapping in patients with stable coronary artery disease: validation by delayed-enhancement magnetic resonance imaging. *Circulation*. 2002;106:957–961.
12. Perin E, Silva GV, Sarmento-Leite R. Left ventricular electromechanical mapping as a diagnostic method. In: Abela GS, ed. *Myocardial Revascularization: Novel Percutaneous Approaches*. New York, NY: Wiley-Liss; 2001:183–195.
13. Jain RK. Molecular regulation of vessel maturation. *Nat Med*. 2003;9:685–693.
14. Carmeliet P. Angiogenesis in health and disease. *Nat Med*. 2003;9:653–660.
15. Carmeliet P. Mechanisms of angiogenesis and arteriogenesis. *Nat Med*. 2000;6:389–395.
16. Isner JM, Asahara T. Angiogenesis and vasculogenesis as therapeutic strategies for postnatal neovascularization. *J Clin Invest*. 1999;103:1231–1236.
17. Oettgen P. Transcriptional regulation of vascular development. *Circ Res*. 2001;89:380–388.
18. Ziegelhoeffer T, Fernandez B, Kostin S, Heil M, Voswinckel R, Helisch A, Schaper W. Bone marrow-derived cells do not incorporate into the adult growing vasculature. *Circ Res*. 2004;94:230–238.

Intracoronary autologous bone-marrow cell transfer after myocardial infarction: the BOOST randomised controlled clinical trial

Kai C Wollert, Gerd P Meyer, Joachim Lotz, Stefanie Ringes-Lichtenberg, Peter Lippolt, Christiane Breidenbach, Stephanie Fichtner, Thomas Korte, Burkhard Hornig, Diethelm Messinger, Lubomir Arseniev, Bernd Hertenstein, Arnold Ganser, Helmut Drexler

Lancet 2004; 364: 141–48

See Comment page 121

Departments of Cardiology and Angiology (K C Wollert MD, G P Meyer MD, S Ringes- Lichtenberg MD, P Lippolt MD, C Breidenbach MD, S Fichtner BS, T Korte MD, B Hornig MD, Prof H Drexler MD), Diagnostic Radiology (J Lotz MD), and Haematology and Oncology (L Arseniev MD, B Hertenstein MD, Prof A Ganser MD), Hanover Medical School, Hanover, Germany; and IST, Mannheim, Germany (D Messinger MSc)

Correspondence to: Prof Helmut Drexler, Abteilung Kardiologie und Angiologie, Medizinische Hochschule Hannover, Carl-Neuberg Strasse 1, 30625 Hannover, Germany drexler.helmut@mh-hannover.de

Summary

Background Emerging evidence suggests that stem cells and progenitor cells derived from bone marrow can be used to improve cardiac function in patients after acute myocardial infarction. In this randomised trial, we aimed to assess whether intracoronary transfer of autologous bone-marrow cells could improve global left-ventricular ejection fraction (LVEF) at 6 months' follow-up.

Methods After successful percutaneous coronary intervention (PCI) for acute ST-segment elevation myocardial infarction, 60 patients were randomly assigned to either a control group (n=30) that received optimum postinfarction medical treatment, or a bone-marrow-cell group (n=30) that received optimum medical treatment and intracoronary transfer of autologous bone-marrow cells 4·8 days (SD 1·3) after PCI. Primary endpoint was global left-ventricular ejection fraction (LVEF) change from baseline to 6 months' follow-up, as determined by cardiac MRI. Image analyses were done by two investigators blinded for treatment assignment. Analysis was per protocol.

Findings Global LVEF at baseline (determined 3·5 days [SD 1·5] after PCI) was 51·3 (9·3%) in controls and 50·0 (10·0%) in the bone-marrow cell group (p=0·59). After 6 months, mean global LVEF had increased by 0·7 percentage points in the control group and 6·7 percentage points in the bone-marrow-cell group (p=0·0026). Transfer of bone-marrow cells enhanced left-ventricular systolic function primarily in myocardial segments adjacent to the infarcted area. Cell transfer did not increase the risk of adverse clinical events, in-stent restenosis, or proarrhythmic effects.

Interpretation Intracoronary transfer of autologous bone-marrow-cells promotes improvement of left-ventricular systolic function in patients after acute myocardial infarction.

Introduction

Rapid reperfusion of the infarct-related coronary artery is of great importance in salvaging ischaemic myocardium and limiting the infarct size in patients with acute myocardial infarction. When done expeditiously and expertly, percutaneous transluminal coronary angioplasty with stent implantation is the method of choice to re-establish coronary flow.[1] Unfortunately, myocardial necrosis starts rapidly after coronary occlusion, usually before reperfusion can be achieved.[2] The loss of viable myocardium initiates a process of adverse left-ventricular remodelling, leading to chamber dilatation and contractile dysfunction in many patients.[3] In this context, much interest has followed from experimental studies showing that cardiac transfer of unfractionated bone-marrow cells, or stem cells and progenitor cells derived from bone marrow can enhance functional recovery after acute myocardial infarction.[4,5] Based on these data, stem cells and progenitor cells derived from bone marrow have been proposed for use in the repair of cardiac tissue after acute myocardial infarction in patients.[6–8]

Early clinical investigations indicate that infusion of autologous bone-marrow cells into the infarct-related coronary artery is feasible after acute myocardial infarction.[9,10] However, because these studies were not randomised trials, the efficacy of intracoronary transfer of bone-marrow cells for functional recovery after acute myocardial infarction in patients has remained uncertain. We did a randomised controlled trial to assess the effect of intracoronary transfer of autologous bone-marrow cells on left-ventricular functional recovery in patients after acute myocardial infarction and successful percutaneous coronary intervention (PCI).

Methods

Patients

Patients were eligible if they were admitted within 5 days of the onset of symptoms of a first ST-segment elevation myocardial infarction, had undergone successful PCI with stent implantation in the infarct-related artery, and had hypokinesia or akinesia involving more than two thirds of the left-ventricular anteroseptal, lateral, and/or inferior wall, as shown by angiography done immediately after PCI. We excluded patients who had multivessel coronary artery disease, pulmonary oedema, cardiogenic shock, advanced renal

or hepatic dysfunction, or documented terminal illness or cancer.

This randomised-controlled study of BOne marrOw transfer to enhance ST-elevation infarct regeneration (the BOOST trial) was approved by our local Ethics Committee. Patients provided written informed consent.

Randomisation and baseline cardiac MRI

Patients were randomly allocated in a 1:1 ratio to either the control or bone-marrow-cell groups, with use of sequentially numbered, sealed envelopes provided by IST (DM). After randomisation, all patients underwent cardiac MRI.

Harvest and transfer of bone-marrow cells

After baseline cardiac MRI, bone marrow was harvested from patients in the bone-marrow-cell group. Bone marrow was processed by 4% gelatine-polysuccinate density gradient sedimentation according to current Good Manufacturing Practice (GMP) regulations (Cytonet, Hannover, Germany), to reduce the volume of the preparation and to deplete erythrocytes and platelets. The final suspension of bone-marrow cells was washed and resuspended in saline with 10 000 U/L heparin.

We used an automated haemocytometer to measure the number of nucleated cells, packed-cell volume, and platelet count in the initial bone marrow aspirate and in the final preparation of bone-marrow cells. Nucleated cell viability was assessed by trypan blue exclusion. We measured the number of CD34+ cells with flow cytometry analysis (FACSCalibur, BD Biosciences, Heidelberg, Germany) using an antibody from Beckman Coulter (Krefeld, Germany). Haemopoietic colony-forming cell growth was measured by a methylcellulose assay (StemCell Technologies, St Katharinen, Germany).

6–8 h after bone-marrow harvest, the final preparation of bone-marrow cells was infused into the infarct-related artery via the central lumen of an over-the-wire balloon catheter (Concerto, Occam International, Eindhoven, Netherlands). To allow bone-marrow cells maximum contact time with the microcirculation of the infarct-related artery, the balloon was inflated inside the stent to transiently interrupt antegrade blood flow during infusions. The entire bone-marrow-cell preparation was infused during four to five coronary occlusions, each lasting 2·5–4 min. Between occlusions, the coronary artery was reperfused for 3 min.

Follow-up

All patients were treated with aspirin (300 mg daily for 4 weeks after PCI, then 100 mg daily), clopidogrel (300 mg loading dose, then 75 mg daily for at least 4 weeks after PCI), an angiotensin-converting enzyme (ACE) inhibitor or angiotensin-receptor blocker, a β blocker, and a statin (if LDL cholesterol concentrations were above 2·6 mmol/L), unless these agents were contraindicated. At both 6 weeks and 3 months after discharge, patients had follow-up examinations to assess their clinical status and to review their current medication. Where necessary, dosages of angiotensin-converting enzyme inhibitors (ACE-inhibitors), angiotensin-receptor blockers, β blockers, and statins were adjusted in accordance with current practice guidelines.[11,12] 6 months after discharge, cardiac MRI was repeated in all patients. In addition, patients were scheduled to undergo coronary angiography to assess the degree of restenosis in the stented segment of the infarct-related artery. Restenosis was quantified with a computer-based system (CMS, Medical Imaging Systems, Leiden, Netherlands) by an investigator unaware of treatment assignment (AM).

To assess whether intracoronary bone-marrow-cell transfer was associated with proarrhythmic effects, we obtained 24 h Holter recordings from all patients before hospital discharge, and at 6 weeks', 3 months', and 6 months' follow-up. From these recordings, the mean number of premature ventricular complexes per h was calculated. We also recorded the number of non-sustained and sustained ventricular tachycardias per recording. In addition, patients were scheduled to undergo programmed ventricular stimulation at 6 months' follow-up. Ventricular stimulation was done at the right-ventricular apex and the right-ventricular outflow tract with single, double, and triple extra stimuli at twice the diastolic threshold and basic cycle lengths of 500 ms and 400 ms.

Cardiac MRI

Cardiac MRI was done with the patient in supine position in a 1.5-T scanner (CV/i, General Electric, Munich, Germany) using electrocardiogram (ECG) gating and a four-element phased array receiver coil. To measure left-ventricular volumes, we used repeated breath-hold fast gradient echo sequences in a steady state (FIESTA, General Electric). Sequence parameters were as follows: TR/TE 3·8/1·6 ms, 40° flip angle, 224×224 matrix, field of view 36–38 cm, in-plane resolution 1·6×1·6–1·7×1·7 mm, 38–40 phases per RR-interval, 10 mm slice thickness. An end-diastolic, horizontal long-axis plane of the left ventricle at end-expiration provided the reference image on which a stack of contiguous short-axis slices was positioned to cover the entire left ventricle.

Contrast-enhanced MRI was used to assess myocardial injury after acute myocardial infarction.[11] A breath-hold k-space segmented T1 weighted inversion recovery gradient echo sequence was used to cover the entire left ventricle with 7–8 mm short-axis slices as

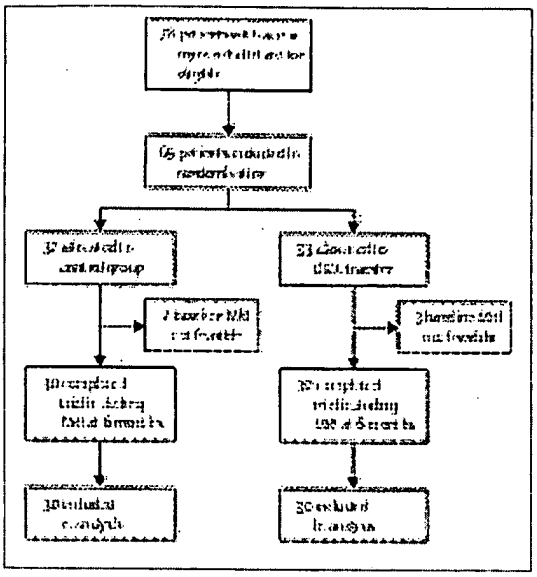


Figure 1: Trial profile
BMC=bone-marrow cell.

described above (TR/TE 7·1/3·1 ms, 256×192 matrix, field of view 36–38 cm, in-plane resolution 1·4×1·9–1·5×2·0 mm). Inversion time (200–220 ms) was individually adapted to null the signal of the myocardium. End-diastolic images were obtained starting 15 min after an intravenous bolus injection of 0·15 mmol/kg gadobutrol, a gadolinium-based extracellular contrast agent (Schering, Berlin, Germany).

All image analyses were done by two investigators who were unaware of treatment assignment (CB and SF), using the MASS 4.0.1 software (Medical Imaging Systems). Endocardial and epicardial borders were traced in all end-diastolic and end-systolic short-axis slices to determine left-ventricular end-diastolic volumes (LVEDV) and end-systolic volumes (LVESV) for global and regional calculation of left-ventricular ejection fraction (LVEF), and left-ventricular mass. For assessment of infarct volumes, late contrast enhancement was quantified. LVEDV index, LVESV index, and left-ventricular-mass index were calculated by dividing LVEDV, LVESV, and left-ventricular mass by body surface area. Regional LVEF was derived by calculating LVEF only in slices showing late contrast enhancement at baseline. Regional left-ventricular function was assessed by determining systolic wall motion in the infarct region and border zone. Systolic-wall motion was defined as the radial displacement of the endocardial contour at systole. Myocardial segments showing late contrast enhancement at baseline were defined as the infarct region. Segments adjacent to the infarct region were defined as the border zone.

Statistical analysis

Primary endpoint was the change from baseline in global LVEF at 6 months' follow-up. Secondary endpoints were changes in LVEDV index, LVESV index, left-ventricular-mass index, and late contrast enhancement. We calculated that we would need

	Control group (n=30)	BMC group (n=30)	p
Age (years)	59·2 (13·5)	53·4 (14·8)	0·11
Men	22 (73%)	20 (67%)	0·57
Body-mass index (kg/m²)	26·2 (4·2)	25·8 (3·0)	0·67
Diabetes mellitus	3 (10%)	3 (10%)	1·0
Hyperlipidaemia*	7	9	0·56
Hypertension	13 (43%)	9 (30%)	0·28
Current cigarette use (number of patients)	17 (57%)	18 (60%)	0·79
Median time from symptom onset to PCI (h) (range)	8·0 (3–120)	9·8 (2–22)	0·92
Killip class			
1	25 (83%)	23 (77%)	0·51
2	5 (17%)	7 (23%)	
3 or 4	0	0	
Infarct-related artery			
Right coronary artery	7 (23%)	7 (23%)	1·0
Left coronary artery	23 (77%)	23 (77%)	
TIMI flow grade			
before PCI:			0·73
Grade 0 or I	16 (53%)	13 (43%)	
Grade II	13 (43%)	16 (53%)	
Grade III	1 (3%)	1 (3%)	
after PCI:			0·75
Grade 0 or I	0	0	
Grade II	7 (23%)	6 (20%)	
Grade III	23 (77%)	24 (80%)	
Maximum serum creatine kinase concentration (U/L)	2844 (1161)	2968 (1867)	0·77
Maximum serum creatine kinase MB concentration (U/L)	156 (51)	175 (123)	0·46
Maximum serum troponin T concentration (μg/L)	7·4 (4·4)	7·4 (5·5)	0·99
Periprocedural therapy			
Thrombolytic therapy before PCI	10 (33%)	14 (47%)	0·29
Platelet glycoprotein IIb/IIIa inhibitors	14 (47%)	14 (47%)	1·0
Median number of stents (range)	1 (1–5)	1 (1–2)	0·40
Size of stent (mm)	3·3 (0·4)	3·3 (0·4)	1·0
Length of stent (mm)	17·5 (9·6)	17·6 (6·4)	0·97
Lesion characteristics			0·71
Type A	8 (27%)	6 (20%)	
Type B	16 (53%)	19 (64%)	
Type C	6 (20%)	5 (3%)	
Medication at primary discharge:			
Aspirin† and clopidogrel	29 (97%)	30 (100%)	
ACE-inhibitors or angiotensin-receptor blockers	30 (100%)	30 (100%)	
β blockers	30 (100%)	29 (97%)	
Statins	29 (97%)	30 (100%)	
at 6 months' follow-up:			
Aspirin†	27 (97%)	29 (97%)	
ACE-inhibitors or angiotensin-receptor blockers	30 (100%)	30 (100%)	
β blockers	30 (100%)	29 (97%)	
Statins	28 (93%)	28 (93%)	

BMC=bone-marrow cell. ACE=angiotensin-converting enzyme. Data are means (SD) or n (%) unless otherwise stated. *Serum cholesterol >5·2 mmol/L. †Patients not receiving aspirin were treated with phenprocoumon.

Table 1: Patients' characteristics

	Baseline		6 months		Change		BMC treatment effect*	p
	Controls	BMC group	Controls	BMC group	Controls	BMC group		
LVEDV index (mL/m²)	81·4 (16·9)	84·2 (17·2)	84·9 (21·9)	91·7 (26·0)	3·4 (11·1)	7·6 (20·0)	4·0 (−4·4 to 12·5)	0·32
LVESV index (mL/m²)	40·6 (16·9)	43·0 (14·7)	42·6 (23·5)	42·4 (23·9)	2·0 (11·1)	−0·6 (14·9)	−3·2 (−9·7 to 3·3)	0·33
Global LVEF (%)	51·3 (9·3)	50·0 (10·0)	52·0 (12·4)	56·7 (12·5)	0·7 (8·1)	6·7 (6·5)	6·0 (2·2 to 9·9)	0·0026
LVM index (g/m²)	78·2 (18·3)	82·7 (18·7)	71·7 (14·2)	71·9 (14·6)	−6·5 (12·8)	−10·8 (10·6)	−2·5 (−7·3 to 2·3)	0·30
LE (mL)	30·3 (17·4)	33·0 (21·1)	19·8 (9·8)	18·9 (12·2)	−10·5 (10·6)	−14·1 (13·0)	−2·2 (−5·4 to 1·0)	0·18

BMC=bone-marrow cell. Data are mean (SD) unless otherwise stated. *Treatment effects expressed as differences in least-squares means (ANCOVA model) with 95% CI. LVM=left ventricular mass. LE=late contrast enhancement. There were no differences between groups at baseline.

Table 2: Left ventricular volume and mass indices, global LVEF, and late enhancement as determined by contrast-enhanced MRI at baseline and 6 months' follow-up

30 patients in each group to achieve a power of at least 80% to detect a difference in global LVEF change of 5 percentage points between study groups, with a two-sided significance level of $p<0·05$, and a common standard deviation of 6·5 percentage points for the global LVEF change from baseline to 6 months' follow-up. We used ANCOVA to compare global LVEF changes in the two study groups, with bone-marrow-cell treatment as the main factor and LVEF at baseline as a covariate. To estimate the treatment effect, differences in least-squares means and corresponding 95% CI were calculated based on the ANCOVA model. We analysed secondary endpoints using the same methods. The consistency of the treatment effect on global LVEF change was assessed across several subgroups. All statistical tests were two-sided with a significance level of $p<0·05$.

Homogeneity of treatment groups at baseline was assessed using Student's t test for continuous variables showing no marked deviations from the normal distribution. For other continuous variables or ordinal baseline data, the Wilcoxon rank-sum test was used. Categorical baseline data were investigated using χ^2 tests. The relation between the number of nucleated cells, CD34+ cells, and haemopoietic colony-forming cells infused into the infarct-related coronary artery and subsequent global LVEF changes were assessed with Pearson's correlation coefficient.

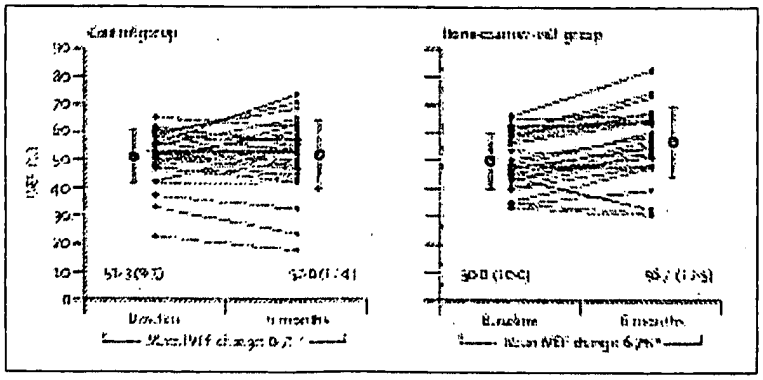


Figure 2: Global LVEF at baseline and 6 months' follow-up
*p=0·0026 for difference between groups. Small dots show data for individual patients; large dots show mean values. Vertical bars show SD.

Subgroup analyses were not prespecified but were exploratory in nature. All subgroup analyses are reported.

Results

Between January, 2002, and May, 2003, 78 patients were informed about the trial. 65 patients were randomly allocated to treatment. After randomisation, five patients were withdrawn because they could not undergo cardiac MRI, either because of claustrophobia or severe obesity. The final cohort included 30 controls and 30 patients in the bone-marrow-cell group (figure 1). Table 1 shows patients' baseline characteristics. All patients received optimum post-infarction medical treatment (table 1).

Mean time from PCI to baseline cardiac MRI was 3·5 days (SD 1·5). Mean time from PCI to bone-marrow harvest was 4·8 days (1·3). Time from symptom onset to harvest of bone-marrow cells was 5·7 days (1·2). On average, 128 mL (33) of bone marrow was aspirated from the posterior iliac crest during a brief general anaesthesia with midazolam and etomidate. No bleeding complications at the harvest site were noted.

During preparation of bone-marrow cells, the sedimentation process reduced the volume of bone-marrow cells to a mean of 26 mL (SD 4) and recovered 75% (12) of nucleated cells from the initial bone-marrow aspirate. The final preparation of bone-marrow cells contained $24·6\times10^8$ (SD $9·4\times10^8$) nucleated cells (viability 99% [2]), $9·5\times10^6$ ($6·3\times10^6$) CD34+ cells, and $3·6\times10^6$ ($3·4\times10^6$) haemopoietic colony-forming cells. The packed cell volume of the final bone-marrow-cell preparation was 31% (11), and the platelet count was 182×10^9 (93×10^9) per mL.

Changes of LVEDV index, LVESV index, left-ventricular-mass index, and late-contrast enhancement from baseline to 6 months' follow-up did not differ significantly between the control and bone-marrow-cell groups (table 2). The increase in LVEDV index at 6 months was slightly higher in the bone-marrow-cell group, whereas LVESV index tended to decrease more in the bone-marrow-cell group (table 2). 6 months after randomisation, global LVEF increased significantly in the bone-marrow-cell group compared with controls

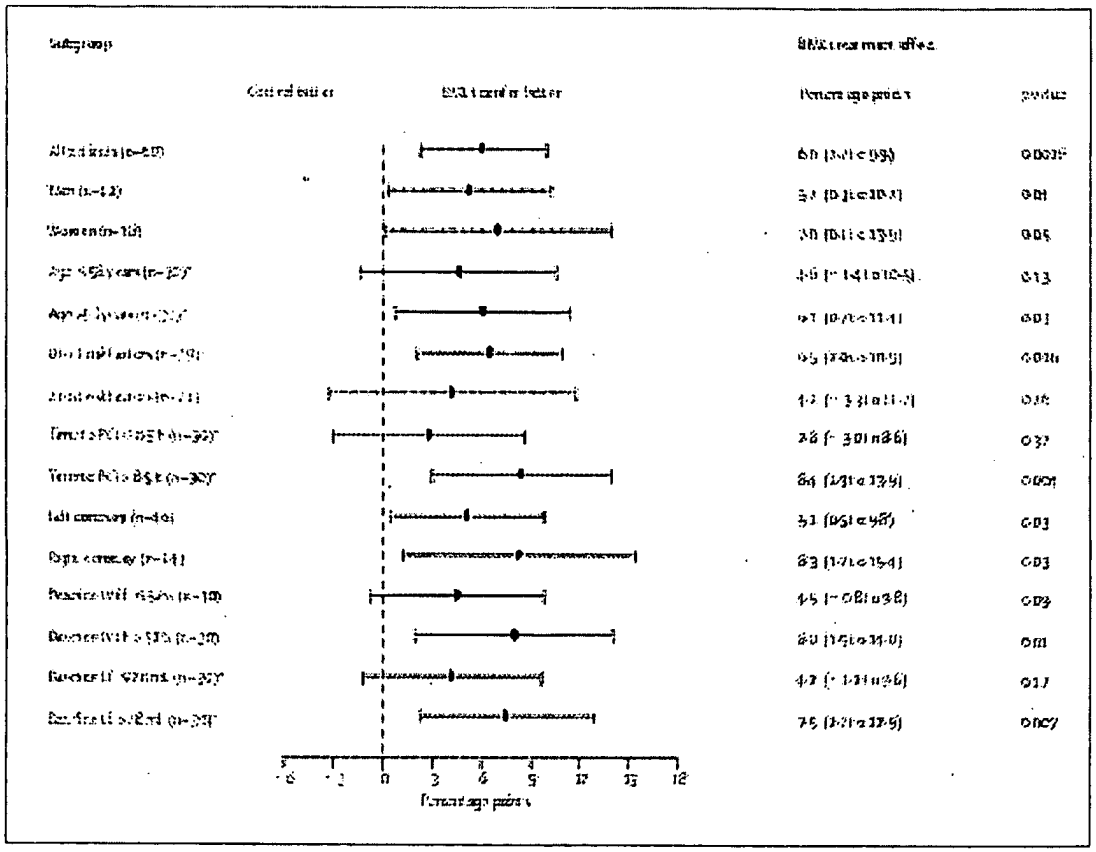


Figure 3: **Subgroup analyses of global LVEF changes from baseline at 6 months' follow-up**
LE=late contrast enhancement. BMC=bone-marrow cell. *Median values of the whole study population were used to create subgroups of equal size. †Cardiovascular risk factors were diabetes, total cholesterol concentration greater than 5·2 mmol/L, hypertension, or current smoking. Oval dots show differences of least-squares means between groups; horizontal bars show 95% CI.

(p=0·0026) (table 2 and figure 2). The effects of bone-marrow-cell transfer on global LVEF change at 6 months' follow-up were consistent in all investigated subgroups (figure 3). The improvement in global LVEF after 6 months' follow-up was not correlated with the number of nucleated cells (r=–0·11, p=0·57), CD34+ cells (r=0·13, p=0·48), or haemopoietic colony-forming cells (r= –0·14, p=0·46) infused into the infarct-related coronary artery.

Compared with the control group, patients in the bone-marrow-cell group had increased regional LVEF (p=0·04) and systolic wall motion in the border zone (p=0·03) at 6 months. By contrast, systolic wall motion in the infarct region was not significantly enhanced by transfer of bone-marrow-cells (table 3). Representative colour-coded images showing the effects of bone-marrow-cell transfer on left-ventricular function are shown in figure 4.

No patient died or was lost to follow-up. There were no increases in troponin T concentrations in serum in any of the patients 24 h after intracoronary transfer of bone-marrow cells, indicating that the procedure did not inflict additional ischaemic damage to the myocardium. In 6 months of follow-up, three controls and one patient from the bone-marrow-cell group needed at least one hospital admission

	Baseline		6 months		Change		BMC treatment effect†	p
	Controls	BMC group	Controls	BMC group	Controls	BMC group		
Regional LVEF (%)	47·8 (9·7)	46·3 (10·6)	48·9 (15·2)	53·0 (15·5)	1·1 (11·8)	6·7 (9·5)	5·7 (0·2 to 11·3)	0·04
Systolic wall motion (mm), infarct region	3·9 (1·8)	4·4 (1·9)	4·9 (2·9)	5·9 (2·5)	1·0 (2·5)	1·5 (2·1)	0·6 (-0·6 to 1·8)	0·32
Systolic wall motion (mm), border zone	6·8 (1·6)	7·0 (1·7)	6·8 (2·1)	8·0 (2·1)	–0·1 (2·2)	1·0 (1·9)	1·1 (0·1 to 2·1)	0·03

BMC=bone-marrow cell. Data are mean (SD). Treatment effects are expressed as differences in least-squares means (ANCOVA model) and 95% CI. There were no differences between groups at baseline.

Table 3: **Regional LVEF and systolic wall motion as determined by contrast-enhanced MRI at baseline and 6 months' follow-up**

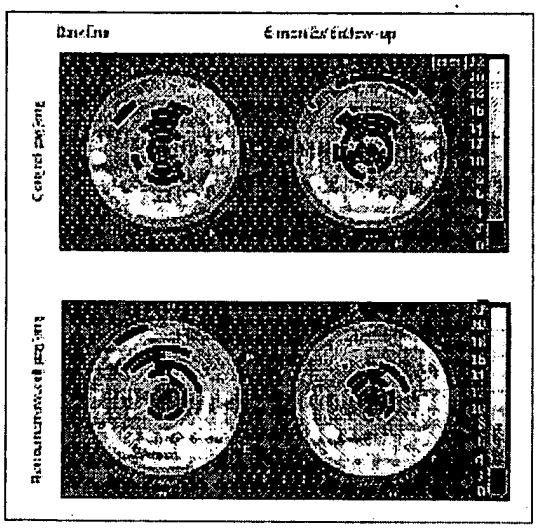


Figure 4: **Representative colour-coded images showing systolic wall motion at baseline and 6 months' follow-up in two patients**
Both patients had had an anterior acute myocardial infarction. Bright colours indicate good systolic wall motion, whereas dark colours indicate poor wall motion (expressed in mm). Note improved functional recovery in the patient treated with bone-marrow-cells.

for worsening heart failure. One person from the bone-marrow-cell group developed a non ST-segment elevation myocardial infarction in the left circumflex territory 4 months after transfer of bone-marrow-cells into the left anterior descending coronary artery. This patient underwent PCI of the left circumflex coronary artery and completed the study.

There were no differences between the control and bone-marrow-cell groups with respect to the number of premature ventricular complexes per h and the occurrence of non-sustained or sustained ventricular tachycardias by Holter monitoring at 6 weeks', 3 months', and 6 months' follow-up. 28 (93%) controls and 27 (90%) patients who had bone-marrow-cell transfer agreed to undergo an electrophysiological study at 6 months' follow-up. A non-sustained ventricular tachycardia was inducible in one control patient and in one bone-marrow-cell transfer patient. Ventricular fibrillation was inducible in one control patient. In 29 (97%) controls and 28 (93%) patients who had bone-marrow-cell transfer, coronary angiograms were obtained at 6 months' follow-up. Mean in-stent restenosis in the infarct-related artery, expressed as a percentage of luminal diameter, was 32% (SD 20) in the control group and 33% (23) in the bone-marrow-cell group (p=0·88). Four patients from the control group and seven from the bone-marrow-cell group presented with an in-stent restenosis of at least 50% (p=0·28). One patient from the control group developed total in-stent occlusion.

Discussion

Our randomised controlled clinical trial addresses the effect of autologous bone-marrow-cell therapy on left-ventricular functional recovery after acute ST-segment elevation myocardial infarction. We have shown that infusion of autologous bone-marrow-cells into the infarct-related coronary artery during the early postinfarction period (4–8 days after symptom onset) improves recovery of global LVEF after 6 months.

In view of the size of our trial, subgroup analyses must be considered with caution. With this caveat in mind, it is noteworthy that the effects of bone-marrow-cell transfer on global LVEF change were consistent across all investigated subgroups. The effects of cell transfer were over and above benefits associated with established strategies to promote functional recovery after acute myocardial infarction, such as PCI with stent implantation, and postinfarction pharmacotherapy with ACE-inhibitors, angiotensin-receptor blockers and β blockers.[11,12]

Global LVEF at baseline was 51% (SD 10) in our patient cohort, which is consistent with previous MRI studies in patients after myocardial infarction.[14,15] In healthy adults, normal LVEF values of 67% (5) have been shown with MRI.[16] Therefore, patients enrolled in our study had substantial functional impairment. Global LVEF increased by only 0·7 percentage points after 6 months' in the control group, emphasising the need for additional therapeutic strategies to enhance functional recovery in patients with acute myocardial infarction. Since 40% of patients had been transferred for rescue PCI from outside hospitals, the average time from symptom onset to PCI was quite long in our trial (median 8·5 h). Previous studies have shown that greater LVEF improvement (up to 4 percentage points) can be achieved when coronary patency is re-established within 4 h of symptom onset.[17,18] Of note, however, is that in these studies baseline LVEF was measured within 24 h of PCI.[17,18] By contrast, we assessed baseline LVEF 3·5 days (SD 1·5) after PCI, at a time when left-ventricular function is likely to have partly recovered from postischaemic myocardial dysfunction (ie, stunning).[19] Similar to the results obtained in our control group, two MRI studies that used serial LVEF measurements in patients with reperfused myocardium after acute myocardial infarction have reported no significant improvement in LVEF from a baseline investigation at day 5–7, to follow-up at 3–6 months.[14,15]

Improvement of global LVEF in the treatment group was due mostly to improved regional systolic wall motion in the infarct border zone. Left-ventricular end-diastolic volumes did not decrease, indicating that transfer of bone-marrow-cells did not improve left-ventricular remodelling at 6 months. Longer follow-up of our patients is required (and will be done) to assess the impact of bone-marrow-cell transfer on long-term

left-ventricular structural adaptation after acute myocardial infarction.

Because of ethical considerations, we decided not to do bone-marrow aspiration and a sham left-heart catheterisation in patients randomised to the control group. Importantly, however, all MRI data were analysed by two investigators who were not aware of treatment assignments.

Our study was not designed to assess underlying mechanisms of treatment with bone-marrow-cells that promote functional recovery after acute myocardial infarction. Apparently, transdifferentiation of bone-marrow-derived haemopoietic stem cells to cardiomyocytes cannot account for the beneficial effects.[20,21] Instead, recent papers have highlighted the potential of bone-marrow cells to promote paracrine effects in ischaemic tissues (eg, secretion of angiogenic factors), and suggest that paracrine signalling, rather than cell incorporation, promotes functional recovery.[5,22–25]

Our experience suggests that intracoronary bone-marrow-cell transfer is safe; specifically, there was no evidence for an increased rate of in-stent restenosis or proarrhythmic effects. It should be noted that high rates of in-stent restenosis have been reported after intracoronary transfer of granulocyte colony-stimulating-factor mobilised peripheral-blood mononuclear cells.[26] Importantly, granulocyte colony-stimulating factor, which may promote in-stent restenosis by enhancing neutrophil recruitment at sites of tissue injury,[27] was not used in our study. Intracoronary injection of bone marrow-derived mesenchymal stromal cells has been shown to cause microinfarctions in dogs.[28] It should be noted that nucleated bone-marrow cells are significantly smaller than expanded mesenchymal stromal cells ex vivo,[28] which may explain why we, and others,[10] did not observe infarctions (ie, increases in concentrations of troponin T in serum) after intracoronary transfer of bone-marrow cells.

Our results lend support to the concept that autologous bone-marrow cells can be used to enhance left-ventricular functional recovery in patients after acute myocardial infarction. Larger trials are needed to address the effect of bone-marrow cell transfer on clinical endpoints such as the incidence of heart failure and survival.

Contributors

K C Wollert contributed to study design, enrolment, and clinical follow-up of patients, aspiration and intracoronary transfer of bone marrow, and the writing of the manuscript. G P Meyer contributed to study design, enrolment of patients, MRI data acquisition, and intracoronary BMC transfer. J Lotz contributed to MRI data acquisition. C Breidenbach and S Fichtner analysed MRI data. S Ringes-Lichtenberg contributed to enrolment and clinical follow-up of patients. T Korte did electrophysiological studies. B Hornig did intracoronary transfer of bone-marrow cells. P Lippolt and D Messinger did statistical analyses. I. Arseniev did bone-marrow-cell sedimentations. B Hertenstein and A Ganser contributed to study design and did bone-marrow aspirations. H Drexler contributed to study design and the writing of the manuscript.

Conflict of interest statement

I. Arseniev is business unit leader of Cytonet Hannover, the company that did the bone-marrow-cell sedimentations during the trial. I. Arseniev has not been involved in any way in MRI data collection or data analysis in this trial.

Acknowledgments

Kai C Wollert and Gerd P Meyer contributed equally to this work. We thank Alix Menke and Dieter Fischer for analysing the coronary angiograms; our colleagues and nurses at the Departments of Cardiology and Angiology and Diagnostic Radiology, and at Cytonet Hannover for their support during the trial. The trial was supported by internal funding from the Department of Cardiology.

References

1 Keeley EC, Boura JA, Grines CL. Primary angioplasty versus intravenous thrombolytic therapy for acute myocardial infarction: a quantitative review of 23 randomised trials. *Lancet* 2003; 361: 13–20.
2 Giugliano RP, Braunwald E. Selecting the best reperfusion strategy in ST-elevation myocardial infarction: it's all a matter of time. *Circulation* 2003; 108: 2828–30.
3 Cohn JN, Ferrari R, Sharpe N. Cardiac remodeling: concepts and clinical implications: a consensus paper from an international forum on cardiac remodeling. *J Am Coll Cardiol* 2000; 35: 569–82.
4 Orlic D, Kajstura J, Chimenti S, et al. Bone marrow cells regenerate infarcted myocardium. *Nature* 2001; 410: 701–05.
5 Kamihata H, Matsubara H, Nishiue T, et al. Implantation of bone marrow mononuclear cells into ischemic myocardium enhances collateral perfusion and regional function via side supply of angioblasts, angiogenic ligands, and cytokines. *Circulation* 2001; 104: 1046–52.
6 Blau HM, Brazelton TR, Weimann JM. The evolving concept of a stem cell: entity or function? *Cell* 2001; 105: 829–41.
7 Korbling M, Estrov Z. Adult stem cells for tissue repair: a new therapeutic concept? *N Engl J Med* 2003; 349: 570–82.
8 Perin EC, Geng YJ, Willerson JT. Adult stem cell therapy in perspective. *Circulation* 2003; 107: 935–38.
9 Strauer BE, Brehm M, Zeus T, et al. Repair of infarcted myocardium by autologous intracoronary mononuclear bone marrow cell transplantation in humans. *Circulation* 2002; 106: 1913–18.
10 Assmus B, Schachinger V, Teupe C, et al. Transplantation of Progenitor Cells and Regeneration Enhancement in Acute Myocardial Infarction (TOPCARE-AMI). *Circulation* 2002; 106: 3009–17.
11 Ryan TJ, Antman EM, Brooks NH, et al. 1999 update: ACC/AHA guidelines for the management of patients with acute myocardial infarction: executive summary and recommendations: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Committee on Management of Acute Myocardial Infarction). *Circulation* 1999; 100: 1016–30.
12 Hunt SA, Baker DW, Chin MH, et al. ACC/AHA guidelines for the evaluation and management of chronic heart failure in the adult: executive summary a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Committee to revise the 1995 guidelines for the evaluation and management of heart failure): developed in collaboration with the international society for heart and lung transplantation; endorsed by the Heart Failure Society of America. *Circulation* 2001; 104: 2996–3007.
13 Wu E, Judd RM, Vargas JD, Klocke FJ, Bonow RO, Kim RJ. Visualisation of presence, location, and transmural extent of healed Q-wave and non-Q-wave myocardial infarction. *Lancet* 2001; 357: 21–28.
14 Schroeder AP, Houlind K, Pedersen EM, Nielsen TT, Egeblad H. Serial magnetic resonance imaging of global and regional left ventricular remodeling during 1 year after acute myocardial infarction. *Cardiology* 2001; 96: 106–14.
15 Beek AM, Kuhl HP, Bondarenko O, et al. Delayed contrast-enhanced magnetic resonance imaging for the prediction of regional functional improvement after acute myocardial infarction. *J Am Coll Cardiol* 2003; 42: 895–901.
16 Lorenz CH, Walker ES, Morgan VL, Klein SS, Graham TP Jr.

See Seminar page 183 for further reading on stem cells and the heart.

Normal human right and left ventricular mass, systolic function, and gender differences by cine magnetic resonance imaging. *J Cardiovasc Magn Reson* 1999; 1: 7–21.

17 Montalescot G, Barragan P, Wittenberg O, et al. Platelet glycoprotein IIb/IIIa inhibition with coronary stenting for acute myocardial infarction. *N Engl J Med* 2001; 344: 1895–903.

18 Stone GW, Grines CL, Cox DA, et al. Comparison of angioplasty with stenting, with or without abciximab, in acute myocardial infarction. *N Engl J Med* 2002; 346: 957–66.

19 Braunwald E, Kloner RA. The stunned myocardium: prolonged, postischemic ventricular dysfunction. *Circulation* 1982; 66: 1146–49.

20 Murry CE, Soonpaa MH, Reinecke H, et al. Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. *Nature* 2004; 428: 664–68.

21 Balsam LB, Wagers AJ, Christensen JL, Kofidis T, Weissman IL, Robbins RC. Haematopoietic stem cells adopt mature haematopoietic fates in ischaemic myocardium. *Nature* 2004; 428: 668–73.

22 Tateishi-Yuyama E, Matsubara H, Murohara T, et al. Therapeutic angiogenesis for patients with limb ischaemia by autologous transplantation of bone-marrow cells: a pilot study and a randomised controlled trial. *Lancet* 2002; 360: 427–35.

23 Ziegelhoeffer T, Fernandez B, Kostin S, et al. Bone marrow-derived cells do not incorporate into the adult growing vasculature. *Circ Res* 2004; 94: 230–38.

24 Kinnaird T, Stabile E, Burnett MS, et al. Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote in vitro and in vivo arteriogenesis through paracrine mechanisms. *Circ Res* 2004; 94: 678–85.

25 Heil M, Ziegelhoeffer T, Mees B, Schaper W. A different outlook on the role of bone marrow stem cells in vascular growth: bone marrow delivers software not hardware. *Circ Res* 2004; 94: 573–74.

26 Kang HJ, Kim HS, Zhang SY, et al. Effects of intracoronary infusion of peripheral blood stem-cells mobilised with granulocyte-colony stimulating factor on left ventricular systolic function and restenosis after coronary stenting in myocardial infarction: the MAGIC cell randomised clinical trial. *Lancet* 2004; 363: 751–56.

27 Chakraborty A, Hentzen ER, Seo SM, Smith CW. Granulocyte colony-stimulating factor promotes adhesion of neutrophils. *Am J Physiol Cell Physiol* 2003; 284: C103–10.

28 Vulliet PR, Greeley M, Halloran SM, MacDonald KA, Kittleson MD. Intra-coronary arterial injection of mesenchymal stromal cells and microinfarction in dogs. *Lancet* 2004; 363: 783–84.

Circulation
JOURNAL OF THE AMERICAN HEART ASSOCIATION

Stem Cell Therapy in Perspective

Bodo E. Strauer and Ran Kornowski

Circulation 2003;107;929-934
DOI: 10.1161/01.CIR.0000057525.13182.24
Circulation is published by the American Heart Association. 7272 Greenville Avenue, Dallas, TX 72514

 Print ISSN: 0009-7322. Online ISSN: 1524-4539

MINI-REVIEW: EXPERT OPINIONS

Stem Cell Therapy in Perspective

Bodo E. Strauer, MD; Ran Kornowski, MD

The concept of regenerative medicine using the body's own stem cells and growth factors to repair tissues may become a reality as new basic science works and initial clinical experiences have "teamed-up" in an effort to develop alternative therapeutic strategies to treat the diseased myocardium. In particular, revealing the signals that mediate cellular growth and differentiation may provide novel tools designed for myocardial regeneration in patients sustaining ischemic cardiomyopathy syndromes. We attempt herein to provide a critical overview of recent developments of myocardial cell transplantation strategies.

Stem Cells

Stem cells are a population of immature tissue precursor cells capable of self-renewal and provision of de novo and/or replacement cells for many tissues. Embryonic stem cells can be obtained from the inner cell mass of the embryonal blastocyst. Although it was recently shown that human embryonic stem cells can differentiate into cardiomyocytes,[1] because of the immunogenicity and rejection, as well as ethical considerations, these cells may be restricted to experimental in vitro studies and their therapeutical potential remains to be determined. Also, these cells may act as an unanticipated arrhythmogenic source after intramyocardial transplantation.[2] Clinical application of these cells is most likely years ahead (Table).

In contrast, adult human stem cells (hematopoietic, mesenchymal) are found in mature tissues, eg, the bone marrow. Plasticity of adult stem cells can probably generate lineages of cells different from their original organ of origin. Thus, these cells can be used for organ regeneration and for cellular repair in various species, as well as in humans.

Ethical problems for adult autologous stem cells do not exist, and although much experimental work remains to be done, their clinical relevance and therapeutic benefit in heart disease have recently been shown for the first time.[3]

Except for hematopoietic and mesenchymal stem cells, many other bone marrow-related cell types may participate in organ repair of infarction models; bone marrow hemangioblasts take part in neovascularization, mesodermal progenitor cells are contained within the mononuclear bone marrow cell fraction that differentiates to endothelial cells, and endothelial progenitor cells can transdifferentiate into cardiomyocytes. Primitive bone marrow cells mobilized by stem cell factor and granulocyte-colony stimulating factor are capable of homing to infarct regions, replicating, differentiating, and promoting myocardial repair.[4] Ultimately, a variety of different cell types from the mononuclear bone marrow cell fraction contribute to the regeneration of necrotic myocardium and damaged vessels. In this regard, therapeutic use of mononuclear cell populations of bone marrow may be more useful and promising than single isolated cell fractions alone. The effect manifested by more heterogenous bone marrow cell populations that contain very small numbers of stem cells may also suggest the importance of an entire array of bone marrow-derived growth factors and cytokines that may also regulate cellular growth and regeneration via cellular secretion mechanisms.

Stem Cells and Angiogenesis

The complex cellular and molecular mechanisms by which endothelial and smooth-muscle cells interact with each other to form blood vessels are now better understood.[5] Endothelial cells alone can initiate the formation and sprouting of

The opinions expressed in this article are not necessarily those of the editors or of the American Heart Association.

From the Department of Medicine, Division of Cardiology, Heinrich-Heine Universitaet Duesseldorf, Duessledorf, Germany (B.E.S.); and Cardiology Department, Rabin Medical Center, Petach-Tikva, Israel (R.K.).

Correspondence to Prof Dr med B.E. Strauer, MD, Department of Medicine, Division of Cardiology, Heinrich-Heine Universitaet Duesseldorf, Moorenstr. 5, 40225 Duessledorf, Germany. E-mail strauer@med.uni-duesseldorf.de

(*Circulation*. 2003;107:929-934.)

Circulation is available at http://www.circulationaha.org

DOI: 10.1161/01.CIR.0000057525.13182.24

Advantages and Disadvantages of Embryonic Versus Adult Stem Cells

	Embryonic Stem Cells	Adult Stem Cells
Advantages	Highly expandable	Easily obtainable
	Pluripotent	No ethical objections
		Different expansion ability
		Uni-, bi-, multi- or pluripotent
		Highly compatible
		Autologous transplantation, no immune-suppressive therapy necessary
		Clinical application already realized
Disadvantages	Ethical objections	Lack of specific identification markers
	Difficult to isolate	
	Risk of rejection	
	Immune-suppressive therapy required	
	Arrhythmogenic potential	
	High risk of teratocarcinomas	
	Clinical application not feasible for 10 to 20 years	
	Lack of specific identification markers	

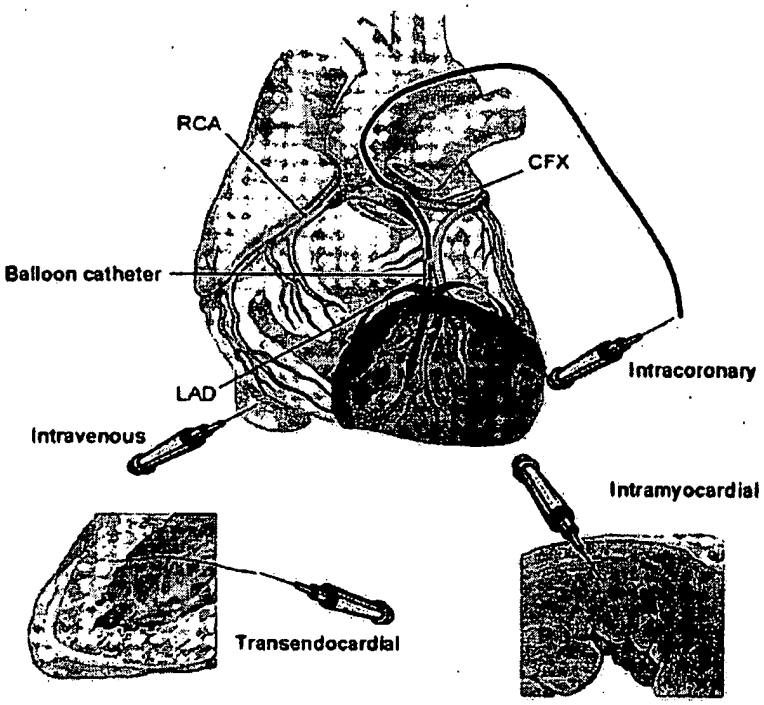
endothelium-lined channels, namely angiogenesis, in response to a physiological or pathological stimulus. Peri-endothelial cells are required for vascular maturation. Recruitment of smooth muscle cells provides these vessels with essential viscoelastic and vasomotor properties and enables accommodating the changing needs in tissue perfusion. This later stage is called arteriogenesis and has a major role in collateral growth.[6] Endothelial progenitor cells could be isolated from peripheral blood and/or bone marrow and showed incorporation into sites of physiological and pathological neovascularization in vivo after either systemic injection or using direct intramyocardial transplantation.[7] In contrast to differentiated endothelial cells, transplantation of progenitor cells successfully enhanced vascular development by in situ differentiation and proliferation within ischemic organs.[8] On the basis of these findings, the beneficial property of endothelial progenitor cells is attractive for angiogenic cellular interventions and as cell-mediated vehicles for gene therapy applications targeting regeneration of ischemic tissue and of failing hearts.

Stem Cell Differentiation to Muscle Cells

The principal aim is to transplant cells of primarily noncardiac origin, such as human bone marrow-derived mononuclear cells containing human stem cells. These cells may operate as a precursor of heart muscle tissue and of coronary blood vessel cells. Human bone marrow contains hematopoietic (1% to 2%) and mesenchymal stem cells (<0.05%). Both types of stem cells may contribute to heart muscle repair. Hematopoietic stem cells are progenitor cells for many types of cells, eg, endothelial cells, which may also differentiate to heart muscle cells. Mesenchymal stem cells are progenitor cells for types of cells such as heart muscle cells, as well as for a variety of cells of noncardiac concern. Recent results in mouse experiments suggest the potency of extracardiac progenitor cells for transdifferentiation into new cardiomyocytes after acute experimental myocardial infarction.[4] Bone marrow cells cultured with 5-azacytidine differentiated into cardiac-like muscle cells in culture and in vivo in ventricular scar tissue in pigs and improved myocardial function.[9] In clinical myocardial infarction, evidence has been provided that autologous bone marrow stem cells may regenerate in infarcted myocardium and improve myocardial perfusion of the infarct zone.[3] Studies with transplanted human hearts have shown that adult humans have extracardiac progenitor cells capable of migrating to and repopulating damaged myocardium, a process occurring at very low levels.[10] Recently, cases have been described in which a male patient receives a heart from a female donor, which provided an opportunity to test whether progenitor cells translocate from the recipient to the graft on the basis of Y chromosome labeling.[11] Results showed that myocytes, coronary arterioles, and capillaries that had a Y chromosome made up 7% to 10% of those in the donor hearts and were proliferative. This indicates a regenerative capacity of the transplanted myocardium. Thus, there is growing evidence for a repair function of extracardiac cells, eg, from bone marrow in the case of cardiac lesion and the necessity of myocardial healing, although these results are not unanimously approved.[12]

Milieu-Dependent Differentiation and Enhanced Environment

Studies from several species demonstrate that bone marrow-derived stem cells are stem cells for various mesenchymal tissues. The cells are therefore not simply stromal precursors, but precursors of peripheral tissues, such as heart muscle.[13] Normal growth and ultimate stem cell fate depend on engraft-



Delivery options for stem cell transfer modalities to the heart. The red colored area represents apical lesion of the left ventricle by myocardial infarction. The balloon catheter is localized in the infarct-related artery and is placed above the border zone of the infarction. Blue and green arrows suggest the possible route of cell infusion and migration into the infarct. The 2 small figures depict the transendocardial and intramyocardial route of administration. RCA indicates right coronary artery; LAD, left anterior descending coronary artery; and CFX, circumflex artery.

ment in an appropriate "niche." Nonetheless, the mechanisms by which the local milieu influences stem cell differentiation are as yet undetermined. Thus, it seems that the fate of bone marrow stem cells is determined by the environment in which they engraft rather than by an intrinsically programmed fate. Therefore, enhancement of functional activity of the specific organ's niche for heart muscle, eg, by positive inotropic (pharmacologic augmentation of contractility) or by positive chronotropic stimuli (heart rate increase by exercise), may promote and intensify the transdifferentiation of bone marrow-derived stem cells to the cardiomyocyte phenotype. After an injury, eg, myocardial infarction, or a cellular damage, eg, in severe pressure or volume overload of the heart, specific factors, including cytokines, stem cell factor, and various growth factors, that stimulate cell replication and substitution in the injured tissue are released by the surrounding cells. In addition, transplanted stem cells, differentiating to cardiomyocytes, become indistinguishable over time from the surrounding cardiomyocytes, and they begin to express the contractile proteins specific for striated heart muscle, including desmin, a-myosin, heavy chain, a-actinin, and phospholamban at levels that are the same as in the host cardiomyocytes.[14] This transdifferentiation process is more pronounced in injured tissue than in healthy organs and may be intensified when the heart as the recipient organ contributes to its enhanced environment by high chronotropic and inotropic activity. Thus, regionally large concentrations of stem cells and increased mechanical activity of the recipient heart muscle may provide a favorable environment for successful engraftment of stem cells after cardiac injury.

Route of Cell Administration

The appropriate route of cell administration to the damaged organ is an essential prerequisite for the success of organ repair (Figure). High cell concentrations within the area of interest and prevention of homing of transplanted cells into other organs are desirable. Therefore, targeted and regional administration and transplantation of cells should be preferred. Below, several special routes of administration are described.

- In regional heart muscle disease, as in myocardial infarction, selective cell delivery by intracoronary catheterization techniques leads to an effective accumulation and concentration of cells within the infarcted zone. This can be realized in humans with bone marrow-derived cells.[15] With intracoronary administration, all cells must pass the infarct and peri-infarct tissue during the immediate first passage. Accordingly, with the intracoronary procedure, the infarct tissue can be enriched with the maximum available number of cells at all times. Further developments of catheterization systems for various clinical studies are needed.
- The transendocardial and transpericardial route of application has been used in large animal experiments[16] and was also recently tested in patients.[17] The main potential advantage of the surgical procedure is injection under visualization, which allows anatomic identification of the target area and even distribution of the injections. The safety and feasibility of catheter-based transendocardial injection was demonstrated in large animal studies,[18] and initial clinical experience in 19 patients using intramyocardial gene trans-

fer showed similar safety profiles.[19] Current clinical experience is limited to one injection system, using electromechanical mapping to generate 3-dimensional left ventricular reconstruction before the injection. Intraventricular catheter manipulation, however, can injure the myocardium, inducing ventricular premature beats and short runs of ventricular tachycardia. In certain cases, this precludes injection to the more arrhythmogenic zones, and it may extend the duration of the procedure and should always be carefully monitored. Each injection catheter is tested for cell biocompatibility to assure no mechanical or functional damage to cells being propelled under pressure through the narrow injection needle. Future developments with steerable transendocardial injection and delivery systems with mapping of the injured zone are needed. Transendocardial injection of autologous bone marrow cells has also been performed as part of several pilot and phase I studies. Safety and feasibility data are still pending and efficacy parameters need large randomized clinical trials.

- The intravenous route of administration is easiest. The main disadvantage, however, is that approximately only 3% of normal cardiac output will flow per minute through the left ventricle, and it is also limited because of transpulmonary first-pass attenuation effect on the cells. Therefore, this administration technique will require many circulation passages to enable infused cells to come into contact with the infarct-related artery. During that time, homing of infused cells to other organs will considerably reduce the number of cells that will populate the infarcted area.
- Some major cell types, such as skeletal myoblasts, have the disadvantage of an emboligenic potency when delivered systemically. Therefore, intramyocardial injection during open-heart surgery has been tested. This procedure has also been used in humans.[20] However, the therapeutic effect is limited because of severe arrhythmogenic complications. Another approach implanted autologous bone marrow cells during open-heart surgery and could show improvement in myocardial perfusion in 3 of 5 treated patients.[21]

Detection of Transplanted Stem Cells

An important clinical problem will be the identification and localization of transplanted autologous stem cells within the injured area of the heart. The transplanted cell or cell population is a single unit in a complex biological network of other cells. Therefore, for both localization and fate mapping of stem cells within the target organ, specific cell markers are desirable. Thus, analysis of stem cell behavior will presume (1) in situ labeling of a single cell or a transplanted cell population or (2) transplantation of already in vitro labeled cells or cell populations. For labeling in animal experiments, retroviral transduction with a marker gene or labeling with thymidine or bromodeoxyuridine (BrdU) have been used. For clinical detection of stem cells, magnetic labeling and in vivo tracking of bone marrow cells by the use of magnetodendrimers or radioactive detection methods may be useful. Myocardial biopsies in humans hardly will be justifiable under these circumstances. Thus, localization and fate mapping of stem cells in the region of myocardial injury will represent an important task for experimental and clinical stem cell research in the future, as well as for the assessment of time course of proliferation in the recipient new cell homes and for the evaluation of proper cell function after full transdifferentiation. First results through the detection of the reporter gene *LacZ*, by identification of β-galactosidase–positive cells in tissue section and chromosome analysis by fluorescence in situ hybridization (FISH) techniques are encouraging.[22]

Stem Cells for Cardiac Wound Repair: A Joint Clinical and Experimental Approach

In the regenerating tissues, stem cells and progenitor cells in the microenvironment both take part in the renewal process. Bone marrow cells injected or mobilized to the damaged myocardium were shown to behave as cardiac stem cells with remarkable plasticity, giving rise to myocytes, endothelial cells, and smooth muscle cells.[23] In the case of human infarcted tissue, autologous bone marrow cells have shown to be highly effective in wound repair in terms of regenerating heart muscle and improving perfusion in the infarcted and border zone area.[24,25] Clinical studies therefore are necessary — in parallel to basic and experimental investigations — analyzing the promising prerequisites for clinical wound repair, preferably the optimum cell administration to the region of interest of the heart, eg, the infarcted tissue, and their optimum concentration and accumulation by different catheter-based techniques.

Moreover, catheter-guided cell transfer to the human heart has the unique advantages of being safe under local anesthesia and during routine cardiac catheterization, being fast, taking between 20 to 40 minutes for the whole procedure, and allowing the administration of bone marrow cells in abundance, selected or non-selected, from bone marrow puncture to the region of interest, which permits a much greater availability of stem cells for the heart than the normal wound healing in various heart diseases or in cardiac transplantation models per se would bring about.[15]

Experimental studies will be needed simultaneously to differentiate between the therapeutically most successful kinds of bone marrow cells:

Global bone marrow containing all mononuclear bone marrow cells or specifically selected subfractions, as isolated cell fractions containing preferably CD34+ or CD34-, CD45-, or AC133+ cells.

Analysis of the transdifferentiation of bone marrow cells to muscle cells and their contribution to the remodeling process in various heart diseases, including cardiac transplantation models.

Cardiac lesions may be multifactorial and include myocardial infarction, myocarditis, cardiomyopathy or cardiac remodeling due to severe pressure, and volume overload. It is

uncertain whether the same therapeutic approach and the same type of cells will be suitable for all of these different diseases. However, organ repair by stem cells represents a general biological mechanism. Thus, it will be one of the future tasks to find the most practical and specific way of evolving and targeting the healing potency of stem cells for selected cardiovascular diseases.

Therapeutic Alternatives in Advanced Heart Failure

Except for pharmacotherapeutics and other measures, the therapy of severe global heart failure and of advanced regional contraction insufficiency is based on nonpharmacological interventions. These are aimed at unloading the heart (cardiac assist device), harmonizing the electrical and mechanical course of contraction and relaxation (ventricular synchronization), restoring ventricular geometry by ventricular size diminution (myocardial left ventricular resection), or abolishing detrimental volume overload in mitral incompetence (repair of the mitral valve).[26,27] The clinical limitations of all of these approaches, which are aimed at reducing systolic wall stress and myocardial oxygen consumption,[28] justify the search for alternative therapeutic options that may beneficially modify the natural course of the disease. By stem cell-derived de novo restoration of damaged cells, replacement of destroyed and scarred tissue with the consecutive improvement of ventricular performance may be possible. It may be speculated that future therapeutical options of combined therapeutical strategies, eg, ventricular resynchronization together with myocardial stem cell repair, may result in additive therapeutical benefit.

Conclusions and Open Questions

Stem cell therapy represents a fascinating new approach for the management of heart diseases. Recent clinical results have shown the feasibility of adult autologous cell therapy in acute myocardial infarction in humans. However, many unresolved questions about experimental and clinical cardiology are still open for future research, especially many basic problems concerning, among others, the following issues:

- The long-term fate of transplanted stem cells in the recipient tissue.
- The ability of transplanted stem cells to find their optimum myocardial "niche."
- The potency of stem cells to transdifferentiate into heart muscle cells.
- The optimal angiogenic milieu needed for transplanted cells in hypoperfused tissue.
- The capability of the recipient tissue to enable an enhanced environment to offer optimum, milieu-dependent differentiation of engrafted cells.
- Specific detection of engrafted cells or cell populations by labeling techniques.
- The optimal time course of availability and application for stem cell replacement therapy in cardiovascular disease.
- The arrhythmogenic potential of implanted cells.
- The specific characterization of the progenitor cells that should be measured to predict therapeutic effect of transplanted cells.
- Development of safe and reproducible catheter-based delivery systems for depositing stem cells to recipient heart muscle.

Additional research is needed o explore the therapeutic merits of cell transplantation techniques while accepting the likelihood that possible adverse side effects may occur. With regard to the clinical practicability, ethical problems, and hazards of immunogenity, actual and future research will focus preferably on adult stem cells, whereas research on embryonic stem cells may emerge presumably into comparable clinical relevance in several years to come.

References

1. Kehat I, Kenyagin-Karsenti D, Snir M, et al. Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. *J Clin Invest.* 2001;108:407–414.
2. Zhang YM, Hartzell C, Narlow M, et al. Stem cell-derived cardiomyocytes demonstrate arrhythmic potential. *Circulation.* 2002;106:1294–1299.
3. Strauer BE, Brehm M, Zeus T, et al. Intrakoronare, humane autologue Stammzelltransplantation zur Myokardregeneration nach Herzinfarkt. *Dtsch Med Wsch.* 2001;126:932–938.
4. Orlic D, Kajstura J, Chimenti S, et al. Bone marrow cells regenerate infarcted myocardium. *Nature.* 2001;410:701–705.
5. Epstein SE, Fuchs S, Zhou YF, et al. Therapeutic interventions for enhancing collateral development by administration of growth factors: basic principles, early results and potential hazards. *Cardiovasc Res.* 2001;49:532–542.
6. Buschmann I, Schaper W. Arteriogenesis versus angiogenesis: two mechanisms of vessel growth. *News Physiol Sci.* 1999;14:121–125.
7. Asahara T, Masuda A, Takahashi T, et al. Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. *Circ Res.* 1999;85:221–228.
8. Kawamoto A, Gwon HC, Iwaguro H, et al. Therapeutic potential of ex vivo expanded endothelial progenitor cells for myocardial ischemia. *Circulation.* 2001;103:634–637.
9. Tomita S, Li RK, Weisel RD, et al. Autologous transplantation of bone marrow cells improves damaged heart function. *Circulation.* 1999;100(suppl II):II247–II256.
10. Laflamme MA, Myerson D, Saffitz JE, et al. Evidence for cardiomyocyte repopulation by extracardiac progenitors in transplanted human hearts. *Circ Res.* 2002;90:634–640.
11. Beltrami AP, Urbanek K, Kajstura J, et al. Evidence that human cardiac myocytes divide after myocardial infarction. *N Engl J Med.* 2001;344:1750–1757.
12. Taylor DA, Hruban R, Rodriguez R, et al Cardiac chimerism as a mechanism for self-repair: does it happen and if so to what degree? *Circulation.* 2002;106:2–4.
13. Pittenger MF, Marshak DR. Mesenchymal stem cells of human adult bone marrow. In Marshak R, Gardner RL, Gottlieb D, eds. *Stem Cell Biology.* New York, NY: Cold Spring Harbor Laboratory Press; 2001:949–973.
14. Toma C, Pittenger MF, Cahill KS. Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. *Circulation.* 2002;105:93–98.
15. Strauer BE, Brehm M, Zeus T, et al. Repair of infarcted myocardium by autologous intracoronary mononuclear bone marrow cell transplantation in humans. *Circulation.* 2002;106:1913–1918.
16. Kornowski R, Fuchs S, Leon MB, et al. Delivery strategies to achieve therapeutic myocardial angiogenesis. *Circulation.* 2000;101:454–458.

17. Fuchs S, Weisz G, Kornowski R, et al. Catheter-based autologous bone marrow myocardial injection in no-option patients with advanced coronary artery disease: a feasibility and safety study. *Circulation.* 2002; 106(suppl II):II655–II656.
18. Fuchs S, Baffour R, Zhou YF, et al. Transendocardial delivery of autologous bone marrow enhances collateral perfusion and regional function in pigs with chronic experimental myocardial ischemia. *J Am Coll Cardiol.* 2001;37:1726–1732.
19. Losordo DW, Vale PR, Hendel RC, et al. Phase 1/2 placebo-controlled, double-blind, dose-escalating trial of myocardial vascular endothelial growth factor 2 gene transfer by catheter delivery in patients with chronic myocardial ischemia. *Circulation.* 2002;105:2012–2018.
20. Menasche B, Hagege AA, Scorsin M. Myoblast transplantation for heart failure. *Lancet.* 2001;357:279–280.
21. Hamano K, Nishida M, Hirata K. Local implantation of autologeous bone marrow cells for therapeutic angiogenesis in patients with ischemic heart disease: clinical trial and preliminary results. *Jpn Circ J.* 2001;65: 845–847.
22. Sinclair A. Genetics 101: cytogenetics and FISH. *CMAJ.* 2002;167: 373–374.
23. Kocher AA, Schuster MD, Szabolcs MJ, et al. Neovascularization of ischemic myocardium by human bone marrow derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function. *Nat Med.* 2001;7:430–436.
24. Orlic D, Kajstura T, Chimenti S,. Mobilized bone marrow cells repair the infarcted heart, improving function and survival. *Proc Natl Acad Sci U S A.* 2001;98:10344–10349.
25. Friedenstein AF. Precursor cells of mechanocytes. *Int Rev Cytol.* 1976; 47:327–355.
26. Hare JM. Cardiac-resynchronization therapy for heart failure. *N Engl J Med.* 2002;346:1902–1905.
27. Gregoric I, Frazier OF, Couto WJ. Surgical treatment of congestive heart failure. *Congest Heart Fail.* 2002;8:214–219.
28. Strauer BE. Myocardial oxygen Consumption in chronic heart disease: role of wall stress, hypertrophy and coronary reserve. *Am J Cardiol.* 1979;44:730–740.

Circulation
JOURNAL OF THE AMERICAN HEART ASSOCIATION

Bone Marrow–Derived Cardiomyocytes Are Present in Adult Human Heart: A Study of Gender-Mismatched Bone Marrow Transplantation Patients

Arjun Deb, Shaohua Wang, Kimberly A. Skelding, Dylan Miller, David Simper and Noel M. Caplice

Circulation 2003;107;1247-1249; originally published online Feb 24, 2003;
DOI: 10.1161/01.CIR.0000061910.39145.F0
Circulation is published by the American Heart Association. 7272 Greenville Avenue, Dallas, TX 72514
 Print ISSN: 0009-7322. Online ISSN: 1524-4539

Bone Marrow–Derived Cardiomyocytes Are Present in Adult Human Heart

A Study of Gender-Mismatched Bone Marrow Transplantation Patients

Arjun Deb, MD; Shaohua Wang, MD; Kimberly A. Skelding, MD; Dylan Miller, MD; David Simper, MD; Noel M. Caplice, MD, PhD

Background—Recent studies have identified cardiomyocytes of extracardiac origin in transplanted human hearts, but the exact origin of these myocyte progenitors is currently unknown.

Methods and Results—Hearts of female subjects (n=4) who had undergone sex-mismatched bone marrow transplantation (BMT) were recovered at autopsy and analyzed for the presence of Y chromosome–positive cardiomyocytes. Four female gender-matched BMT subjects served as controls. Fluorescence in situ hybridization (FISH) for the Y chromosome was performed on paraffin-embedded sections to identify cells of bone marrow origin with concomitant immunofluorescent labeling for α-sarcomeric actin to identify cardiomyocytes. A total of 160 000 cardiomyocyte nuclei were analyzed approximating 20 000 nuclei per patient. The mean percentage of Y chromosome–positive cardiomyocytes in patients with sex-mismatched BMT was 0.23±0.06%. Not a single Y chromosome–positive cardiomyocyte was identified in any of the control patients. Immunofluorescent costaining for laminin and chromosomal ploidy analysis with FISH showed no evidence of either pseudonuclei or cell fusion in any of the chimeric cardiac myocytes identified.

Conclusions—These data establish for the first time human bone marrow as a source of extracardiac progenitor cells capable of de novo cardiomyocyte formation. (***Circulation.*** **2003;107:1247-1249.)**

Key Words: chimera ■ stem cells ■ myocytes, cardiac ■ transplantation, bone marrow

The concept of the human heart as an organ incapable of self-renewal has recently been challenged by identification of cardiac myocytes of probable extracardiac origin in hearts of patients undergoing sex-mismatched cardiac transplantation.[1–4] The exact source of these cells is currently unclear, but data from experiments in animals support a bone marrow origin.[5] It is important to note that a marked discrepancy in the level of cardiac chimerism has been observed in the gender-mismatched cardiac transplantation setting.[1–4 6,7] Moreover, controversy has arisen with regard to the methodologies used to define chimeric cardiac myocytes in these human studies. Specifically, concerns have recently been raised about the most appropriate techniques required to differentiate (1) true cardiac myocyte nuclei from pseudonuclei,[6] and (2) diploid nuclei from epigenetic phenomena, such as spontaneous cell fusion.[8]

To address the above issues, we used a specific study design and experimental approach. An ideal method to answer the question of bone marrow origin of chimeric myocytes is to analyze hearts of patients who have undergone gender-mismatched bone marrow transplantation (BMT). The presence of Y chromosome–positive cardiomyocytes in the hearts of female patients would conclusively suggest a bone marrow origin for these cells. By using fluorescence in situ hybridization (FISH) combined with immunohistochemistry, we determined the percentage of male cardiomyocytes in autopsy hearts of female patients who had undergone gender-mismatched BMT. To exclude the possibility of false identification of pseudo or fusion nuclei as chimeric cardiomyocytes, additional analyses were performed with the use of basement membrane laminin costaining and chromosome 18 multiploidy analysis with FISH, respectively. Gender-matched BMT patients served as controls.

Methods

Patients and Autopsy Tissue

A review of the Mayo Clinic BMT database and autopsy records identified 4 female patients who had received male donor bone marrow. Female patients who had gender-matched BMT were examined as controls. The Mayo Clinic institutional review board granted approval for the study of human tissue samples.

Combined Immunohistochemical and FISH Analysis

Immunohistochemical analysis of cardiac tissue sections was performed by using a monoclonal antibody against α-sarcomeric actin (Sigma clone 5c5) and a rabbit antibody against laminin (Sigma, St Louis, Mo). The secondary detection used was respectively an anti-mouse antibody conjugated to Cy-3 (Molecular Probes; red) and an anti-rabbit antibody conjugated to Alexa Fluor (Molecular Probes; green). In separate experiments, liver and skeletal muscle tissue from the same subjects was stained with antibodies to human hepatocyte and skeletal muscle actin with the use of monoclonal antibodies

Received November 13, 2002; revision received January 23, 2002; accepted January 23, 2002.
From the Division of Cardiovascular Diseases and Molecular Medicine Program, Mayo Clinic, Rochester, Minn.
Correspondence to Noel M. Caplice, MD, PhD, Mayo Clinic, GU 1801, 200 First St SW, Rochester, MN 55905. E-mail caplice.noel@mayo.edu

Circulation is available at http://www.circulationaha.org

DOI: 10.1161/01.CIR.0000061910.39145.F0

Clinical Data and Cardiomyocyte Chimerism Analysis of Gender-Mismatched BMT Patients

Patient	Age, y	No. Days From Transplantation to Death	Primary Disease	Cause of BMT Death	Ejection Fraction, %	No. of Y Chromosome–Positive Cardiomyocytes (% Chimerism)		Nuclei Counted
1	32	35	CLL	ARDS	67	60	(0.30)	20 106
2	46	510	CML	BOOP	65	35	(0.17)	20 054
3	44	600	CML	Sudden death	66	51	(0.25)	20 109
4	41	480	ALL	GVHD, BOOP	72	36	(0.18)	20 049
Mean±SD	...	...	...	...	...	0.23±0.06%		...

ARDS indicates adult respiratory distress syndrome; BOOP, bronchiolitis obliterans and organizing pneumonia; GVHD, graft vs host disease; CLL, chronic lymphocytic leukemia; CML, chronic myelogenous leukemia; and ALL, acute lymphocytic leukemia.

(both from Dako). Hepatocytes and skeletal myocytes were visualized using a secondary anti-mouse antibody conjugated to Cy-3.

After immunostaining, FISH was immediately performed as previously described.[3] The X and Y chromosome (CEP X, Y; Vysis Inc; B7322, B-6927) DNA probes used were specific for the α satellite region of each chromosome and labeled with Cy-3 and fluorescein isothiocyanate, respectively. For combined analysis, sarcomeric actin and laminin staining and FISH for Y-chromosome were used. In separate experiments a probe to the centromere of human chromosome 18 (CEP 18 Aqua; light blue dot; Vysis) was combined with X (red dot) and Y chromosome (green dot) analysis to evaluate cell ploidy and exclude cell fusion in the chimeric nuclei identified.

In all cases, FISH signals were enumerated using a Zeiss Axioplan microscope equipped with a triple-pass filter (Vysis). Rigorous criteria were used to identify Y chromosome–positive cardiac myocytes as previously described.[2] Counting of the nuclei and Y chromosome was performed by two independent blinded observers.

Results

Patient Characteristics

The clinical profiles of the 4 female patients who underwent sex-mismatched BMT are shown in the Table. Subjects had a range of hematologic diseases requiring BMT ($2.8\pm0.5\times10^8$ infused cells/kg body weight) and received the same pretransplantation conditioning regimen, which consisted of total body irradiation and cyclophosphamide. All patients were maintained on prednisone, and 2 subjects were maintained on additional cyclosporine and azathioprine after transplantation. Autopsy examination showed no macroscopic or microscopic evidence of inflammation in any of the hearts studied (Figure, A).

Immunofluorescence and FISH Analysis

Histological sections of the left ventricle in gender-mismatched subjects revealed a mean percentage of Y chromosome–positive cardiac myocytes of 0.23±0.06% (Table). The Y chromosome was located eccentrically within the nuclei of chimeric cardiomyocytes (Figure, B and C), and chromosomal ploidy analysis excluded cell fusion (Figure, B, inset). Four female control patients who had undergone sex-matched BMT showed no evidence of Y chromosome positivity in any of the 80 000 cardiomyocyte nuclei analyzed. Basement membrane laminin and sarcomeric actin costaining distinguished true chimeric nuclei with surrounding myocyte cytoplasm from pseudonuclei (Figure, C and D). Male bone marrow–derived hepatocytes and skeletal myocytes were also found in the liver and muscle of female gender-mismatched BMT recipients (Figure, E and F), and mean donor cell chimerism in these tissues was 0.4% and 0.2%, respectively (3000 nuclei analyzed). The detection sensitivity of FISH for Y chromosome in this study was 45%, similar to that cited in previous FISH analysis of tissue sections.[2,4]

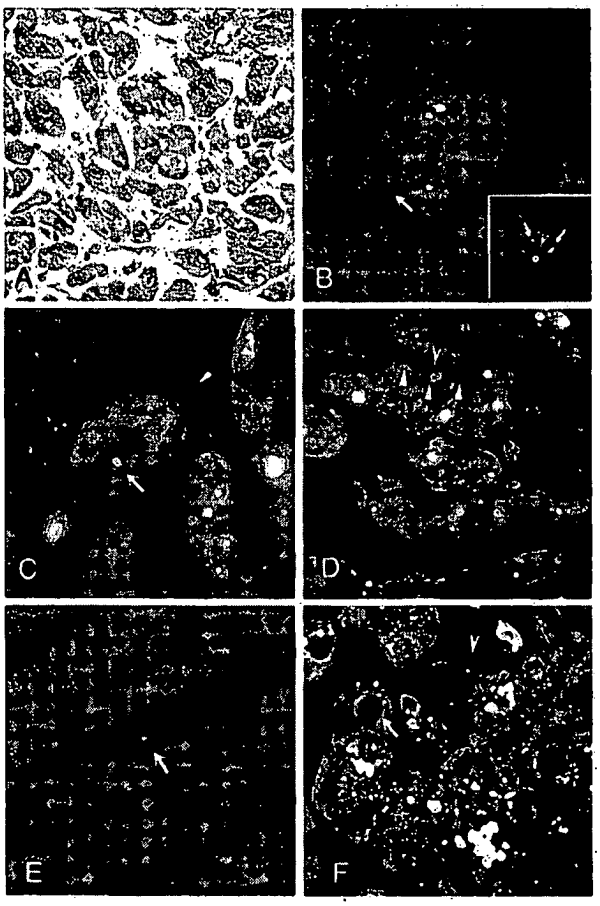
Discussion

These data suggest that adult human bone marrow acts as a source of extracardiac progenitor cells contributing to cardiomyocyte formation. The additional use of laminin costaining and chromosomal ploidy analysis in this study makes the possibility of confusing pseudonuclei or cell fusion events for chimeric myocytes unlikely. The potential origin and phenotype of marrow myocyte precursors in our subjects includes lineage-restricted mesenchymal,[9] hematopoietic,[10] and multipotent adult progenitors[9] and cells of angioblastic lineage.[11]

Physiological stress and tissue injury are known to release cytokines and chemokines, which may promote mobilization of progenitor cells from the bone marrow to the peripheral circulation.[12] Although no patients in our study group had histological evidence of myocardial inflammation, 3 of 4 patients had respiratory complications such as adult respiratory distress syndrome and bronchiolitis obliterans. It is possible that severe tissue injury occurring in these conditions resulted in high levels of circulating cytokines with consequent mobilization of circulating progenitor cells. Interestingly, prior animal experiments showed no detectable engraftment of marrow-derived cells in the absence of myocardial injury.[5] The difference between these animal data and our study may reflect differences in species, duration of study, use of "side population" cells exclusively versus unfractionated bone marrow, or other poorly understood phenomena associated with clinical disease and its treatment.

The consistent levels of chimerism seen at 5 weeks and 20 months after marrow transplantation in our present study suggest a steady-state recruitment of marrow progenitors rather than an initial seeding event early after transplantation. It is noteworthy that a similar recruitment of bone marrow cells occurred in the liver and skeletal muscle as well as the heart, which validates previous animal and human data suggesting multipotent differentiation potential for bone marrow–derived cells.[11,13] It is well known that marrow-derived mesenchymal and hematopoietic stem cells circulate for long periods after transplantation, allowing an equilibrium to be established between circulating and tissue-specific seeding compartments. It is therefore conceivable that low-level recruitment of blood-borne precursors into the myocardium occurs in response to local events in the tissue microenvironment.

Another possibility is that myocardial injury secondary to the pretransplantation conditioning regimen leads by a repair response to recruitment of marrow precursors into the myocardium. This



A, Hematoxylin-and-eosin staining of normal left ventricular myocytes showing no evidence of inflammatory cell infiltrate. B, Cardiomyocyte of female gender-mismatched BMT patient staining positive for α-sarcomeric actin (red) possessing nuclei (blue) positive for Y chromosome (green dot). B, inset, Diploid bone marrow–derived cardiomyocyte nucleus of female gender-mismatched BMT patient showing X chromosome (open arrowhead, red dot), Y chromosome (green dot), and a pair of chromosome 18 (filled arrows, light blue dots) signals; note overlying and surrounding red staining for α-sarcomeric actin. C, Y chromosome–positive true nucleus (blue, green dot; arrow) of bone marrow–derived cardiomyocyte cytoplasm (sarcomeric actin, red) surrounded by basement membrane laminin (green, arrowhead). D, Y chromosome–positive pseudonucleus (open arrowhead) separated from cardiomyocyte (sarcomeric actin, red) by laminin (green-filled arrowheads). E and F, Combined immunofluorescence staining and FISH for Y chromosome in female gender-mismatched BMT subjects showing (E) male skeletal muscle cell (red cytoplasm and blue nucleus with green dot-arrow) and (F) male hepatocyte (red cytoplasm and blue nucleus with green dot-arrow). Note a male cell (open arrowhead) that does not costain with hepatocyte antibody.

scenario seems less likely, however, as the degree of chimerism would be expected to decrease over time and a concurrent "response to injury" would be expected from other blood-borne cells such as leukocytes, neither of which was seen in our study. Furthermore, because all our patients had established hematologic disease before BMT, we cannot automatically infer that chimeric events seen in our study occur under normal healthy conditions, nor can we exclude the possibility that pretransplantation disease may have altered posttransplantation seeding of circulating cells. Finally, we can only speculate on the additional modulating effects of immunosuppressive therapy on bone marrow cell recruitment in our subjects.

The mean percentage of bone marrow–derived cardiac myocytes observed in our subjects was low. It is difficult if not impossible to compare our data with previous chimerism studies both from a clinical and methodological perspective[1–4,7] because it is likely that variables such as chimeric cell detection method, time of study after transplantation, and the presence or absence of inflammation influence the level of myocyte chimerism observed. Finally, while this manuscript was under review, Thiele et al[14] reported 6.4% cardiomyocyte chimerism in a group of male bone marrow transplantation patients, a level more than an order of magnitude greater than our findings. However, the small number of nuclei analyzed and the use of morphology instead of myocyte-specific staining make the identification of chimeric nuclei as true cardiomyocytes less certain in this study.

In conclusion, the present study establishes bone marrow as a contributor to low-level de novo cardiac myocyte formation. The clinical significance of this finding in terms of myocardial regeneration will depend on the success of future efforts to understand and augment the mobilization, homing, and differentiation properties of these cells. Further investigation may also determine whether these cells can be engineered or targeted to diseased myocardium for therapeutic effect.

Acknowledgment

This work was supported in part by grants from the National Institutes of Health (HL66958-NMC) and Mayo Foundation.

References

1. Hruban RH, Long PP, Perlman EJ, et al. Fluorescence in situ hybridization for the Y-chromosome can be used to detect cells of recipient origin in allografted hearts following cardiac transplantation. *Am J Pathol.* 1993;142:975–980.
2. Laflamme MA, Myerson D, Saffitz JE, et al. Evidence for cardiomyocyte repopulation by extracardiac progenitors in transplanted human hearts. *Circ Res.* 2002;90:634–640.
3. Muller P, Pfeiffer P, Koglin J, et al. Cardiomyocytes of noncardiac origin in myocardial biopsies of human transplanted hearts. *Circulation.* 2002;106:31–35.
4. Quaini F, Urbanek K, Beltrami AP, et al. Chimerism of the transplanted heart. *N Engl J Med.* 2002;346:5–15.
5. Jackson KA, Majka SM, Wang H, et al. Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells. *J Clin Invest.* 2001;107:1395–1402.
6. Taylor DA, Hruban R, Rodriguez ER, et al. Cardiac chimerism as a mechanism for self-repair: does it happen and if so to what degree? *Circulation.* 2002;106:2–4.
7. Glaser R, Lu MM, Narula N, et al. Smooth muscle cells, but not myocytes, of host origin in transplanted human hearts. *Circulation.* 2002;106:17–19.
8. Terada N, Hamazaki T, Oka M, et al. Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion. *Nature.* 2002;416:542–545.
9. Jiang Y, Jahagirdar BN, Reinhardt RL, et al. Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature.* 2002;418:41–49.
10. Orlic D, Kajstura J, Chimenti S, et al. Bone marrow cells regenerate infarcted myocardium. *Nature.* 2001;410:701–705.
11. Korbling M, Katz RL, Khanna A, et al. Hepatocytes and epithelial cells of donor origin in recipients of peripheral-blood stem cells. *N Engl J Med.* 2002;346:738–746.
12. Dominko T, Takahashi D, Martinovich C, et al. Ischemia- and cytokine-induced mobilization of bone marrow–derived endothelial progenitor cells for neovascularization. *Nat Med.* 1999;5:431–433.
13. Krause DS, Theise ND, Collector MI, et al. Multi-organ, multi-lineage engraftment by a single bone marrow–derived stem cell. *Cell.* 2001;105:369–377.
14. Thiele J, Varus E, Wickenhauser C, et al. Chimerism of cardiomyocytes and endothelial cells after allogeneic bone marrow transplantation in chronic myeloid leukemia: an autopsy study. *Pathologe.* 2002;23:405–410.